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A Study of the Effects of Sodium Monofluorophosphate

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A STUDY OF THE EFFECTS OF SODIUM MONOFLUOROPHOSPHATE ON THE ENZYMES PHOSPHORYLASE AND PHOSPHATASE

by

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LIFE

Robert Aloysius Sliwinski was born in Joliet, Illinois, on February 5, 1928.

He was graduated from Fenwick High School, Oak Park, Illinois. From September, 1946 to June, 1948, he attended St. Benedict's College, Atchison, Kansas. From September, 1948 to June, 1950, he attended St. Louis University from which he graduated with the degree of Bachelor of Science. He began work for his Master of Science degree in the Graduate School of Loyola University in September, 1950.

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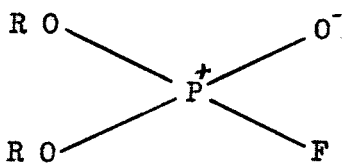
CHAPTER I

INTRODUCTION

Fluorophosphates have recently become of biological importance in that they have a noted effect on enzymes. They have been found to inhibit some of these biological catalysts. Considerable attention has been paid in recent years to the alkyl fluorophosphates, the first of which was investigated by Adrian, Feldberg, and Kilby (1). The inhibition is progressive and irreversible (45) and said to be selective for the non-specific esterase (7,26).

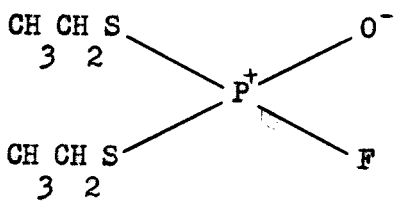
Lange and v. Krueger (38) directed attention to the strong action that traces of the vapors of dialkyl monofluorophosphates have on the human organism. A few minutes after inhaling very small quantities, a choking pressure on the larynx is observed, which is accompanied by difficulties in breathing and followed by disturbances of consciousness and vision and by painful hypersensitivity to light. These effects disappear very slowly even after brief exposure to the ester vapors. They were ascribed to the esters themselves and not to their products of hydrolysis.

In 1932, Lange and v. Krueger (38) described dialkyl esters of monofluorophosphoric acid with the structural formula:



After the outbreak of World War II, the British Ministry of Supply began a study of highly toxic compounds that might have possible military significance. Adrian, Feldberg, and Kilby in May, 1940 (2) were prompted by the above discussed observations to test the effects of the dimethyl and diethyl esters on animals as lethal inhalants. They found that the esters had an extremely high toxicity; and also observed that the diisopropyl ester, commonly abbreviated DFP, prepared about a year later by McCombie and Saunders (47), had an even more powerful effect.

In addition to DFP, the monofluorophosphoric acid diesters of n-propyl, n-butyl, sec-butyl, n-amyl, iso-amyl, sec-hexyl, cyclohexyl, and aryl (e.g. phenyl) were prepared by McCombie, Saunders, Chapman, and Heap (47) and Chapman and Saunders (9), as new compounds. The isopropyl or sec-butyl esters were found to display a much higher toxicity than esters of primary alcohols. The phenyl group reduces toxicity. The typical physiological effects disappear when the ester oxygen atoms are replaced with sulfur.



The discovery of the extreme toxicity of dialkyl monofluorophosphates in 1940 by Adrian, et al (2) was followed in the next year by the discovery by McCombie and Saunders (48a,b) that the toxicity of the dimethyl ester is the result of its cholinesterase inhibiting power, which results

in rapid accumulation of acetylcholine liberated in nerves by impulses. After the more toxic DFP ester had been prepared, Barrett, Feldberg, Kilby, and Kilby (4), Dixon and Needham (15), and Mackworth and Webb (41) found it to have the same action. Dixon and Needham (15) recognized that DFP is one of the most specific and most powerful enzyme inhibitors known. They observed that its toxicity in animals by vapor inhalation is greater than that of phosgene, cyanogene chloride, or picrin, and comparable to hydrogen cyanide. When two men were exposed for three minutes to a concentration of 82 mgs. of DFP per cu. m. (1/100,000), strong effects on the human organism, of the type described by Lange and v. Krueger (38) were observed by Kilby and Kilby (35). When animals were exposed for ten minutes to higher concentrations, severe effects were produced and death frequently resulted, usually within half an hour after beginning of the exposure and sometimes during exposure.

Mazur (46) found an enzyme in rabbit and human plasma, red blood cells, and tissue which accelerates greatly the hydrolysis of dialkyl fluorophosphates. It is not related to phosphatase, cholinesterase, or esterase and is important in the detoxification of DFP in the intact animal. Thus, the reaction of the dialkyl fluorophosphates in the animal organism is a resultant of the irreversible inactivation of cholinesterases and the detoxification of the fluoroester by the new enzyme. Recently, Jansen, Nutting, and Balls (32a) reported the inhibition of plant acetylesterase by DFP. A similar inhibition of acetylesterase from cobra

venom was observed by Bovet-Nitti (8). Webb (62) showed that the alkyl fluorophosphates are not specific inhibitors of cholinesterase but of a whole group of esterases. While the pharmacological action of DFP is usually attributed to its inhibition of cholinesterase, Gerard and co-workers (17, 18) have recently shown that DFP is a powerful dehydrogenase inhibitor as well. They pointed out that its ability to alter physiological behavior of nerve and brain follows its action on respiration more closely than its action on cholinesterase.

Comroe and co-workers (10) found DFP effective to a certain extent in myasthenia gravis, a condition characterized by extreme muscular weakness. In glaucoma, a disease of the eyes caused by increased fluid pressure in the eyeballs, Leopold and Comroe (39a,b) used the ester effectively in a number of cases where all other drugs had failed. Wood (63) used DFP in combination with either prostigmine or pitressin with spectacular success in the severest cases of paralytic ileus, a condition in which the motility of the intestine is lost and the bowel distends with gas.

Successful investigations by Jansen, Nutting, and Balls (32b) have led to a considerable knowledge of the mode of DFP inhibition of enzymes. They found the inhibition of acetylesterase to be bimolecular and independent of the pH of the reaction mixture over the range of stability of the enzyme, and they concluded that the point of attack of the DFP must involve the site of attachment of the enzyme to the substrate. While dialysis of inhibited, purified acetylesterase failed to cause any regeneration of activity,

regeneration of citrus acetylesterase inhibited in situ by DFP was observed, when the intact fruit was allowed to stand 3 to 4 days.

Jansen, Nutting, Jang, and Balls (33) noted that DFP inhibits trypsin and chymotrypsin besides cholinesterase. Its esterase and proteinase activities are equally affected indicating that the two activities probably reside in the same active centers of the enzyme molecule. Approximately one mole of DFP is required for the complete inhibition of one mole of chymotrypsin. By the use of DFP containing radioactive P^{32}, Jansen, Nutting, and Balls (32c) have shown that the phosphorous of DFP is introduced into chymotrypsin, when the enzyme is inhibited by DFP; and the quantity of introduced phosphorous determined in the recrystallized reaction product, corresponds to the quantity of DFP necessary for the complete inhibition of chymotrypsin.

Studies on the inorganic monofluorophosphates and their action on enzymes have been less extensive than the studies of the more complex organic monofluorophosphates and their action on enzymes. First of all, attention should be directed to the fact that the so-called fluorophosphates of the older literature are not true fluorophosphates with anions containing the P-F bond, but phosphates, which, as stated by Seifert (57a,b), instead of $P(OH_3)FOK$, should be written as $KH_2PO_4 \cdot HF$.

A simple method for producing the alkali salts has been described by Hill and Audrieth (28) and by Anderson (3). Anhydrous alkali metaphosphates and fluorides, when fused together, react to form the crude

monofluoro salts in very good yields in accord with the equation:

$$M'PO_3 - M'F = M'_2PO_3F.$$

Sodium monofluorophosphate which is of most interest in this thesis is produced by fusing a mixture of sodium metaphosphate and sodium fluoride in stoichiometric proportions in a closed vessel from which moist air is excluded, and cooling the mass to room temperature before opening the vessel. Sodium pyro- or orthophosphate can be substituted for sodium metaphosphate, but it must first be dehydrated at 300°C. to eliminate combined water. The sodium fluoride can be precipitated by heating equivalent amounts of sodium acid fluoride and sodium carbonate to drive off the carbon dioxide and water formed.

Sodium monofluorophosphate is readily soluble in water. The solution is neutral toward phenolphthalein but alkaline to methyl orange. As with all phosphoric acids, monofluorophosphoric acid has one strong acid hydrogen while the second hydrogen is less easily dissociated and is weaker. Neutral or weakly alkaline solutions, in the absence of cations which form more difficultly soluble phosphates or fluorides, are not hydrolyzed on boiling for one hour and consequently display a remarkable stability, whereas fluoroborates and fluorosilicates, because of partial hydrolysis, react acidic almost immediately after being dissolved in water. If sodium monofluorophosphate is heated in a strongly acidic solution, it is decomposed within a few minutes (52). The sodium salt seems to form a hydrate on crystallization from very cold solutions as indicated by Lange (37) and the

anhydrous compound melts at about 625°C.

Knowledge concerning the pharmacology of monofluorophosphates is very limited. Dixon and Needham (15) found the ammonium salt gives a 50% inhibition of cholinesterase activity when applied in a concentration of 10^{-2}M. The sodium or potassium salts inhibit, or retard the growth and reproduction of a number of molds at concentrations from 0.01 to 0.1 per cent. Interperitoneal injection of a solution of 100 mg. of sodium monofluorophosphate per kilogram of body weight into rats did not indicate any toxicity, whereas a quantity of about 350 mg. appeared to be lethal for the animals (52).

Hodge, Hein, and Shourie (29) determined the ability of monofluorophosphate to inhibit dental caries. By using hamsters as a means of testing this effect as compared to the effect of sodium fluoride, oral and interperitoneal toxicity was determined. Comparison showed sodium monofluorophosphate to be 7 to 8 times less toxic than sodium fluoride. On the basis of fluoride content the complex fluoride is 2.3 to 3 times less toxic than the free ionic form.

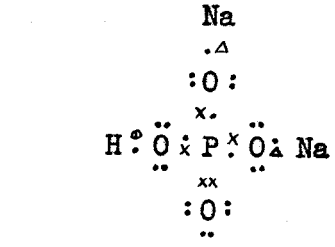
Hence, the alkali monofluorophosphates do not exhibit the exceedingly high toxicity observed with dialkyl monofluorophosphates. Further progress in the studies of monofluorophosphates may be significant not only for a complete knowledge of the action of these compounds, but also for an understanding of the mode of action of certain enzymes.

CHAPTER II

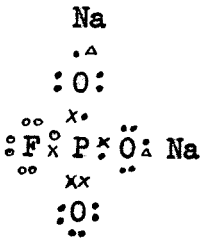
STATEMENT OF THE PROBLEM

In view of what has been stated it has been shown that the relatively large dialkyl monofluorophosphates are highly toxic, whereas the comparatively small inorganic monofluorophosphates are considerably less toxic. The problem of this thesis involves the study of one of the small alkali monofluorophosphates e.g. sodium monofluorophosphate. This study will involve the problem of determining what effect sodium monofluorophosphate might have on certain biological catalysts. The enzymes phosphorylase and phosphatase were chosen because orthophosphate itself is a part of the equilibrium reactions which are catalyzed by these enzymes.

Monofluorophosphate, by nature of its chemical and electronic structure, is very closely related to phosphate, as is shown by the following molecular structures of the sodium salts of each.



Molecular weight = 141.982



Molecular weight = 143.974

FIGURE 1

The molecular weights of each are practically identical. In monofluoro-

phosphate a-hydroxyl group is replaced by a fluoride radical. Because of this close relationship it has been speculated that the monofluorophosphate radical could possibly interfere with enzyme catalyzed reactions involving orthophosphate. To clarify this a diagram would be most appropriate.

$$\text{Starch or glycogen} + \text{orthophosphate} \overset{\text{Phosphorylase}}{\rightleftharpoons} \text{glucose-1-phosphate}$$

If orthophosphate is replaced with monofluorophosphate, will the reaction rate by decreased, or unchanged, or would the enzyme be inactivated? Then if sodium monofluorophosphate is found to be an inhibitor, the mechanism of this inhibition will be studied, that is, will sodium monofluorophosphate inhibit on a competitive or non-competitive basis.

The usefulness of the studies outlined derives not only from the fact that it has the academic value of enlarging our knowledge of the concept of structural competition in enzyme inhibition as a mechanism of drug action, but also from the practical importance of enabling us to use a drug to prevent carbohydrate metabolism in certain restricted areas of the body.

CHAPTER III

ORTHOPHOSPHATE DETERMINATION IN THE PRESENCE OF MONOFLUOROPHOSPHATE

The determination of orthophosphate in the presence of monofluorophosphate was one of the first essential steps which had to be determined because most of the methods for determining the activity of phosphatase and phosphorylase involve the determination of the orthophosphate which had been liberated from or fixated to the substrate. For example, phosphatase activity is commonly determined by allowing the phosphatase solution to hydrolyze some phosphate ester in the presence of a suitable buffer at a fixed temperature for a definite time interval. An aliquot of the digest is then analyzed for free orthophosphoric acid by some such method as that of Sumner (59) or the more complicated method of King and Delory (36).

The method chosen for determining orthophosphate was that of Sumner (59). The method consisted of placing the phosphate sample in a 100 ml. flask. Ten ml. of 6.6% ammonium molybdate were added, followed by water to about the 75 ml. mark. Ten ml. of 7.5 N. sulfuric acid were added and then 8 ml. of 10% ferrous sulfate. The solution was diluted to the 100 ml. mark and allowed to stand for 10 minutes. The intensity of the blue color which developed because of the presence of phosphate was determined colorimetrically by reading the per cent transmission in the Junior Coleman Spectrophotometer (Model 6B) at a wave length of 660 mμ. A reagent blank (a solution prepared in the same manner but with the omission of any

phosphate) was set at 100% transmission. The unknown orthophosphate concentration as determined was compared against a standard curve which was prepared according to the following procedure. A standard solution of potassium dihydrogen phosphate (analytical reagent) was made by first drying the KH_2PO_4 in an oven overnight at 110°C. To a 1 liter flask 2.1964 gm. of KH_2PO_4 were added and diluted to volume with water. This standard solution contained 0.5000 mg. phosphorous per ml. To 100 ml. flasks, 0.1, 0.2, 0.3, etc., to 1.4 ml. of the standard potassium orthophosphate solution were added, corresponding respectively to 0.05, 0.10, 0.15, etc., to 0.70 mg. of phosphorous. Sumner's (59) procedure for determining phosphorous was carried out and a standard curve was drawn on semi-log paper, plotting mg. of phosphorous versus per cent transmission (Figure 2).

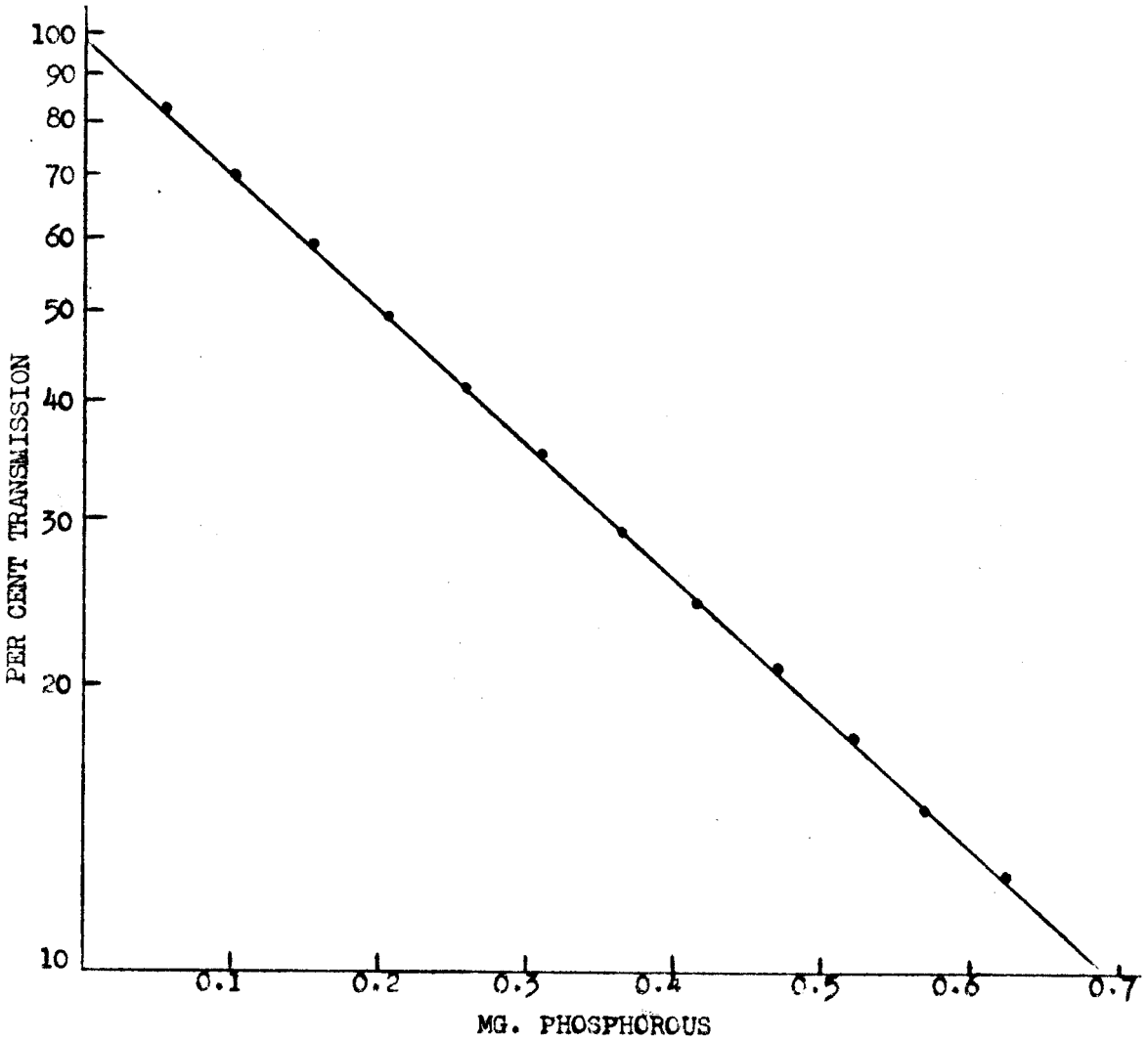
A suitable method for the determination of orthophosphate in the presence of monofluorophosphate could not be found. The general methods for determining orthophosphate could not be used accurately because it had been found that monofluorophosphate is very unstable in the acid medium which is used for all phosphate determinations. The hydrolysis of monofluorophosphate is exceedingly rapid in any solution other than a neutral solution (52). It will be noted from Table I (Part A-1) that 1 ml. of 0.01 M. Na_2HPO_4, which as calculated should contain 0.3098 mg. of phosphorous had been determined colorimetrically to contain 0.3000 mg. Likewise, 1 ml. 0.01 M. Na_2PO_3F (Ozark-Mahoning Co.), which had also been calculated

to contain 0.3098 mg. of phosphorous (Table I, part A-3), only shows a content of 0.0450 mg. as determined colorimetrically. This small amount of orthophosphate is likely to be the amount which had been hydrolyzed from the monofluorophosphate during the 10 minute period which is required for color development in the orthophosphate determination. As is shown in part A-4 of Table I, if the color transmission is read after periods longer than the 10 minutes needed for the color to develop, an increase in orthophosphate is noted for the sodium monofluorophosphate determination, whereas for the sodium phosphate, the orthophosphate determined remains stable for at least 6 hours (Table I, part A-2). This proves that it is not the reagents which cause an increase in color, but rather the hydrolysis of sodium monofluorophosphate in the acid medium of the reagents.

As is also shown in Table I (Part B-2) sodium monofluorophosphate is almost completely hydrolyzed in a 1 N. HCl solution after 7 minutes at 100°C.

For this reason it is very difficult to obtain reproduceable results for determining orthophosphate in the presence of monofluorophosphate. Also, monofluorophosphate cannot be determined by any method which requires an acid medium for the color development.

It has also been found that barium acetate which is one of the most common means of precipitating orthophosphate will also precipitate monofluorophosphate (Table II). To determine this the following method was used. To 10 ml. of sodium monofluorophosphate (0.1 M.), 1 gram of barium



STANDARD CURVE FOR PHOSPHOROUS DETERMINATION

FIGURE 2

TABLE I

THE DETERMINATION OF MONOFLUOROPHOSPHATE

		Time In Min. Which Color Was Determined After Addition of Color Reagents.	Mg. Phosphorous As Calculated	% Transmission	Mg. Phosphorous Determined
A-1.	1 ml. 0.01 M. Na_2HPO_4	10	0.3098	36.5	0.3000
A-2.	1 ml. 0.01 M. Na_2HPO_4	10	0.3098	36.5	0.3000
		30	0.3098	36.5	0.3000
		90	0.3098	36.5	0.3000
		180	0.3098	36.5	0.3000
		270	0.3098	36.5	0.3000
		360	0.3098	36.5	0.3000
A-3.	1 ml. 0.01 M. Na_2PO_3F	10	0.3098	85.0	0.0450
A-4.	1 ml. 0.01 M. Na_2PO_3F	10	0.3098	85.0	0.0450
		30	0.3098	78.0	0.0725
		90	0.3098	68.5	0.1100
		180	0.3098	57.0	0.1650
		270	0.3098	50.0	0.2050
		360	0.3098	47.5	0.2200
B-1.	2 ml. 0.01 M. Na_2HPO_4 heated for 7 min. at 100°C. with 2 ml. 2 N. HCl. A 2 ml. aliquot was used for the phosphorous determination.	10	0.3098	36.0	0.3050
B-2.	2 ml. 0.01 M. Na_2HPO_4 heated for 7 min. at 100°C. with 2 ml. 2 N. HCl. A 2 ml. aliquot was used for the phosphorous determination.	10	0.3098	38.0	0.2800

acetate was added and the mixture stirred vigorously for 3 to 5 minutes. The precipitate (in this case, it is thought to be barium monofluorophosphate) was centrifuged down. To a 2 ml. aliquot of the supernatant, 2 ml. of 2 N. HCl was added to make the total solution 1 N. with respect to hydrochloric acid. The acid solution was heated for 7 minutes at 100°C. The orthophosphate determination test was run on a 2 ml. aliquot. Theoretically, if barium would not precipitate out the monofluorophosphate, the amount of orthophosphate hydrolyzed from the monofluorophosphate in the acid solution at 100°C. for 7 minutes would be equivalent to 0.3098 mg. for the 2 ml. aliquot used, but as the data indicates, the barium does precipitate monofluorophosphate. The same procedure was followed using orthophosphate instead of monofluorophosphate. As was indicated from the experiment (Table II) barium acetate precipitates almost identically as much monofluorophosphate as it does orthophosphate. Thus, it is thought that barium forms a complex with monofluorophosphate in the same manner as it does with orthophosphate, and because of this, barium acetate could be used to precipitate monofluorophosphate from solution as well as orthophosphate.

TABLE II

THE PRECIPITATION OF MONOFLUOROPHOSPHATE

	Test Mixture	Mg. Of Phosphorous As Calculated	% Transmission	Mg. Phosphorous Determined
1.	1 ml. 0.01 M. Na_2PO_3F. Same as Table I, part A-3	0.3098	85.0	0.0450
2.	1 ml. 0.01 M. Na_2PO_3F and acid hydrolysis. Same as Table I, part B-2	0.3098	38.0	0.2800
3.	1 ml. 0.01 M. Na_2PO_3F with barium precipitation and acid hydrolysis	0.3098	88.3	0.0320 90% of initial phosphate precipitated out with barium
4.	1 ml. 0.01 M. Na_2HPO_4. Same as Table I, part A-1	0.3098	36.5	0.3000
5.	1 ml. 0.01 M. Na_2HPO_4 and acid hydrolysis. Same as Table I, part B-1	0.3098	36.0	0.3050
6.	1 ml. 0.01 M. Na_2HPO_4 with barium precipitation and acid hydrolysis	0.3098	90.0	0.0250 92% of initial phosphate precipitated out with barium

CHAPTER IV

PHOSPHORYLASE

History

As early as 1899, Cremer (14) observed that yeast juice is capable of glycogen synthesis. Newberg and Pollok (51) demonstrated in 1910 that sucrose may be changed by phosphorolysis using autolyzed yeast. Cori and Cori (11) were the first to show that muscle tissue and muscle extracts form glucose-1-phosphate from glycogen. Because of this, the ester is called "Cori ester." In more recent years, other enzymes synthesizing various carbohydrates and their derivatives by phosphorylation have been described.

The reversible reaction: Starch + orthophosphate $\xrightleftharpoons{\text{Phosphorylase}}$ glucose-1-phosphate, as catalyzed by phosphorylase, was found to take place as a result of the phosphorolytic breakdown of successive terminal glucose molecules from the starch molecule, without the interaction of water. When the reverse reaction takes place and starch is formed from glucose-1-phosphate, a dephosphorylation with a simultaneous condensation takes place. This phosphorolytic process whereby starch is broken down to glucose with the simultaneous entrance of inorganic phosphate at the α-glucoside linkages resulting in the formation of glucose-1-phosphate is diagramatically represented by Cori, Colowick, and Cori (59) as shown in Figure 3. This scheme applies to glycogen as well as starch.

Parnas (53) called this type of starch breakdown "phosphorolysis"

in order to differentiate it from hydrolysis, which indicates interaction with water.

Both potatoe and muscle phosphorylase are capable of degrading synthetic or natural amylose almost completely. According to Swanson (60), potatoe phosphorylase degrades amylopectin and glycogen to the extent of about 40%. Katz et al (34) found that approximately 90% of amylose is converted to glucose-1-phosphate by this enzyme. Only about 57% of amylopectin is converted to this ester under these conditions. It is suggested that, like B-amylase, potatoe phosphorylase acts upon the branched amylopectin attacking nonreducing ends, splitting off successive terminal glucose fragments (β-amylase splits off maltose), until it encounters a 1, 6-glucosidic linkage at the branching point that acts as an obstruction (20, 21, 49).

Cori and Cori (12) demonstrated that the synthesis of polysaccharide is autocatalytic, since no reaction takes place when highly purified enzyme and chemically synthesized glucose-1-phosphate are used unless a priming agent, such as starch or glycogen, is added. However, for the phosphorolysis of polysaccharide, no glucose-1-phosphate is needed. Crude potatoe phosphorylase and glucose-1-phosphate prepared enzymatically from starch usually contain sufficient dextrin impurities to start the formation of polysaccharide.

According to Cori, Swanson, and Cori (13), the rate of polysaccharide synthesis depends on the nature as well as the amount of

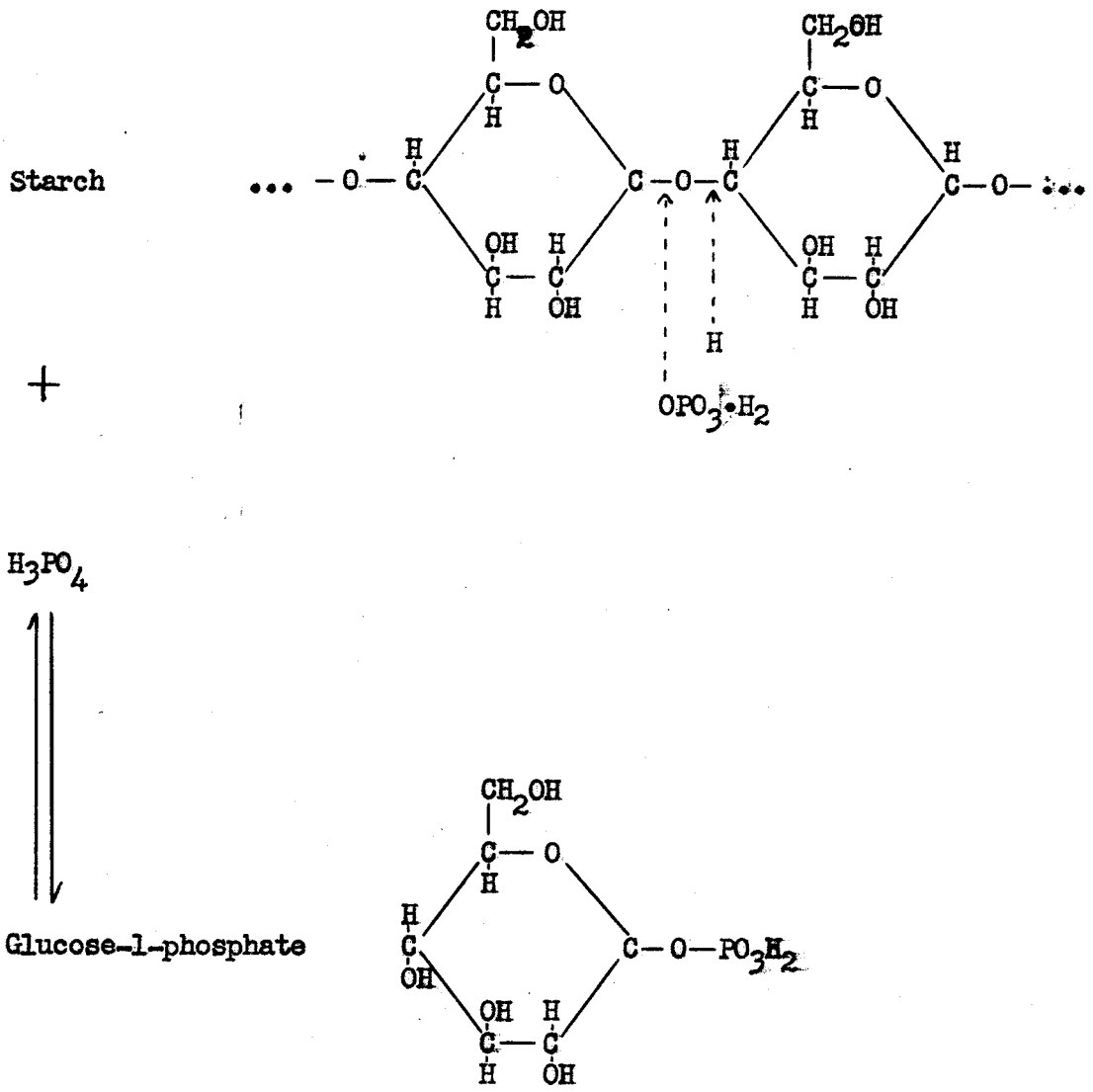


FIGURE 3

activating polysaccharide added to the reaction mixture of glucose-1-phosphate and muscle phosphorylase. Only the branched component of natural starch (amylopectin) can serve as a priming agent. The linear component (amylose) has little effect in concentrations in which amylopectin possesses strong activating power. Amylopectin appears to have a greater activating effect for potatoe phosphorylase, whereas glycogen has a greater effect for muscle phosphorylase.

Cori et al (13) explain the kinetics of polysaccharide synthesis as follows: the catalytic polysaccharide or priming agent (glycogen, starch, or dextrin) can be visualized as a central core or nucleus with a large number of branches or side chains, each averaging from 6 to 25 glucose units in length. These chains are lengthened by the addition of glucose units through the agency of phosphorylase by a repetition of the following process:

$$(C_6H_{10}O_5)_x + C_6H_{11}O_5OPO_3H_2 \rightleftharpoons (C_6H_{10}O_5)_{x+1} + \text{ortho-phosphate.}$$

Activating Polysaccharide glucose-1-phosphate

$$(C_6H_{10}O_5)_{x+1} + C_6H_{11}O_5OPO_3H_2 \rightleftharpoons (C_6H_{10}O_5)_{x+2} + \text{ortho-phosphate.}$$

The theory that the action of phosphorylase consists in lengthening the existing polysaccharide is supported by an experiment of Sumner and collaborators (58). When small, intermediate, and large amounts of a dextrin were added to potatoe phosphorylase as a priming agent, it was observed that at the time an equal quantity of glucose-1-phosphate had reacted, the respective iodine colors were blue, red, and colorless, in-

dicating that the chain length of the newly formed polysaccharide is inversely proportional to the number of end groups added with the priming agent. It, therefore, follows that when the amount of primer is in large excess relative to glucose-1-phosphate, chain length at equilibrium will be an inverse relation to the amount of primer used. If the primer concentration is made very low, it can be expected that long chains will be synthesized. But ultimately, the chain length apparently becomes a limiting factor, and when the chain attains a certain maximum value, the reaction cannot proceed further.

Phosphorylase is specific with regard to its action upon glucose-1-phosphate. Only α-D-glucose-1-phosphate has been found capable of being converted by phosphorylases to polysaccharide. None of the other sugar esters can be substituted for glucose-1-phosphate. Thus β-D-glucose-1-phosphate, α-L-glucose-1-phosphate, maltose-1-phosphate, and D-xylose-1-phosphate could not be polymerized to polysaccharide by potatoe phosphorylase as shown by Hassid and co-workers (22, 23). Hassid et al (24, 25) also showed that both pure potatoe phosphorylase and crystalline muscle phosphorylase produced from glucose-1-phosphate a nonbranched polysaccharide containing only α-1, 4-glucosidic linkages and resembling the amylose fraction of starch. Cori and Bear (5, 6) found that the synthetic polysaccharides, like amylose, are slightly soluble in water, rapidly retrograde from solution on standing and produce a sharp X-ray, V-diffraction pattern. They give a very intense blue color with iodine,

are completely converted to maltose by β-amylase, and have a low activating power on muscle phosphorylase. Data obtained by the end group method showed that, like amylose, the synthetic starches are made up of linear chains of glucopyranose units. Muscle tissue, which is rich in glycogen, contains no amylose; potatoe tubers produce starch consisting predominately of amylopectin. It is clear that purified phosphorylases fail to reproduce the pattern of synthesis characteristic of the intact cells. The assumption has therefore been made that muscle and plant tissues contain a factor different from the isolated phosphorylase, which is specifically responsible for the formation of the branch points, the α-1, 6-linkages.

Preparation of Potatoe Phosphorylase

The enzyme used in these tests was obtained from potatoes. Commercial preparations of potatoe phosphorylase (Delta Chemical Works) were obtained, but after receiving three inactive preparations, it was decided upon to prepare the enzyme in the laboratory. Various methods for purifying potatoe phosphorylase were tried but the activity decreased rapidly. Bunji Maruo (44) devised a method of precipitating the enzyme with dioxane. This was tried without success. Finally, a combination of the methods of Green and Stumpf (19) and Hidy and Day (27) was used with success.

The following method of preparation was used. A sufficient quantity of potatoes (Idaho) to furnish one liter of juice was peeled and sliced and kept under water until used, preferably within one hour. The

slices were drained quickly and pulped in a Waring blendor. The minced material was filtered through cheese cloth. To eliminate both the α- and β-amylase, Weibull and Tiselius (61) adjust the potatoe juice with acetic acid to pH 5.2, add 2.5 gm. kaolin per 100 ml. juice and allow to stand for 10 minutes. The mixture was then centrifuged and the supernatant liquid adjusted to pH 6.5 with dilute ammonium hydroxide. Solid ammonium sulfate was added to bring the specific gravity of the solution to 1.085. The precipitate was centrifuged within half an hour and discarded. The supernatant liquid was raised to a specific gravity of 1.152 with solid ammonium sulfate and then centrifuged. The precipitate, which was not entirely soluble in water, was suspended in 250 ml. of water and fractionation was repeated in a specific gravity range of 1.095 to 1.145. The precipitated enzyme was dissolved in 100 ml. water for two subsequent fractionations in the specific gravity ranges of 1.100 to 1.140 and 1.100 to 1.35 respectively. The pH was determined at frequent intervals throughout the preparation and was kept between 6.0 and 6.5 by the addition of dilute ammonium hydroxide. Rapid inactivation of the enzyme occurs below pH 5.8. The final enzyme precipitate was dissolved in 100 ml. water and stored at $0^{\circ}C$.

It is important that the freshly expressed juice is quickly separated from the pulp, as inactivation is particularly rapid at this stage. All the other steps in the procedure should be carried out as quickly as possible.

A 1 ml. portion of this enzyme solution was found to contain 2.3 units of phosphorylase activity according to the method of Green and Stumpf (19) as will be shown later in this chapter.

Standard Curve for the Starch-Iodine Method of Determining Phosphorylase Activity

The method used for determining phosphorylase activity by starch synthesis is a modification of the method used by Huggins, and Russell (30) for the determination of amylase activity. It is based on the blue starch-iodine color which in an excess of iodine was found to be strictly proportional to the amount of starch present. A plot of the logarithm of the colorimeter reading against the arithmetic concentration of starch in the presence of a constant amount of iodine gives a straight line. By using the curve as a reference standard, the amount of starch present in an unknown sample may readily be determined to within 0.01 mg.

Up to 4 mg. of starch with 4 ml. of 0.01 N. iodine reagent will give colors which when diluted to 100 ml. can be conveniently read in the Junior Coleman Spectrophotometer. A wave length of 660 millimicrons eliminates all extraneous color such as that due to the excess iodine and to the dextrins formed in amylolytic hydrolysis. In this range, a 100 per cent change in iodine concentration has only a slight effect on the color. In this way, measurement of the color given with iodine by aliquot samples of the starch-enzyme mixture before and after incubation permits enzyme activity to be expressed in terms of milligrams of substrate phosphorolyzed. The reverse may also be applicable, that is, the amount of

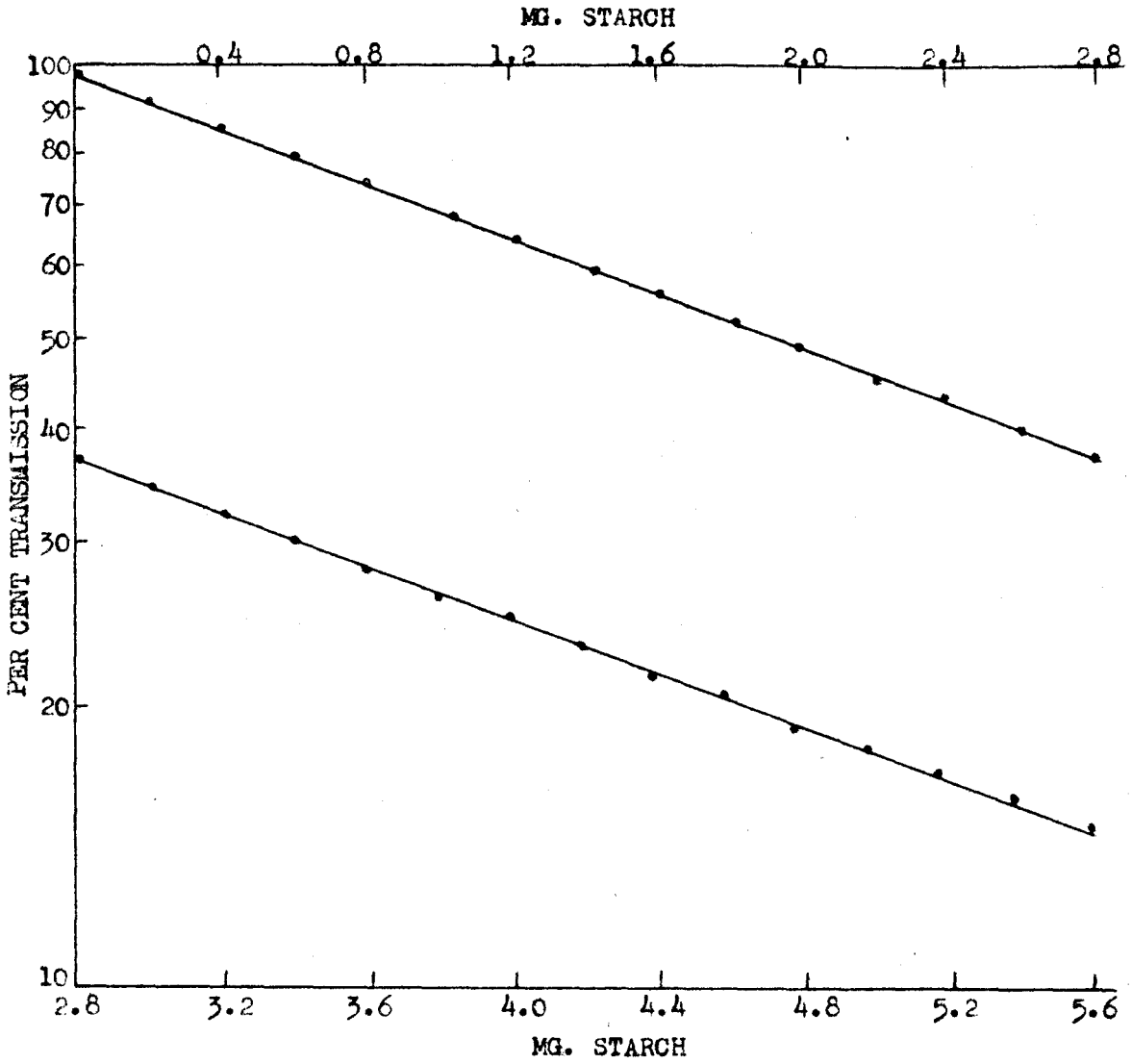
starch formed from certain substrates which snythesize starch may be an expression of enzyme activity. At neutrality the starch-iodine color is stable for many hours both in the dark and in the light.

The standard starch-iodine curve was developed by delivering progressive amounts of starch from 0.1 to 5.6 mg. into each of a series of 100 ml. volumetric flasks containing 4 ml. of 0.01 N. iodine reagent. Each flask is diluted to the mark with water and a sample of the contents is read in the Junior Coleman Spectrophotometer (Model 6B).

The colorimetric readings plotted logarithmically against starch content will give a straight line which was used as the standard curve in all determinations.(Figure 4).

Phosphorylase Determination by the Starch-Iodine Method

The following procedure was used to determine phosphorylase activity by the amount of starch synthesized from glucose-1-phosphate. To a solution containing 4 ml. of veronal buffer (pH 7.0) (50), 2 ml. of 1% glucose-1-phosphate and 0.1 ml. of 5% starch, was added 1 ml. of phosphorylase solution, the final volume being 10 ml. The enzyme solution was added to the rest of the mixture after a temperature equilibrium of 38°C. was attained. The starch was added as a priming agent as was indicated by Cori and Cori (12). The activity of the phosphorylase was measured by the starch-iodine method just described. From the data obtained, it was found that 1 ml. of the phosphorylase solution was able to synthesize 12.95 mg. of starch in 30 minutes from the test solution (Figure 5).



STANDARD CURVE FOR STARCH DETERMINATION

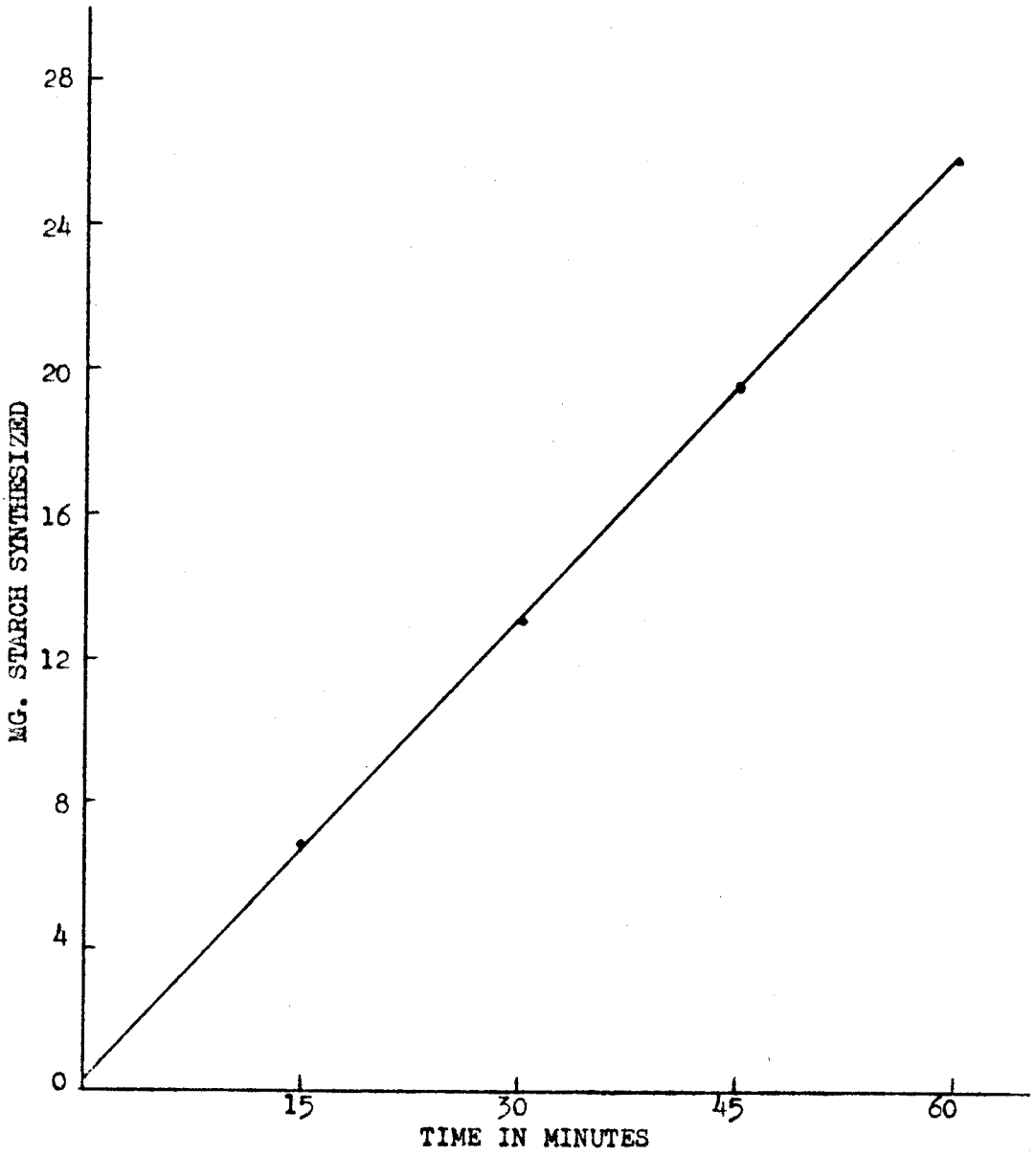
FIGURE 4

A Determination of Amylase as a Contamination of the Phosphorylase Solution

A separate series of test were run on the phosphorylase solution to determine whether amylase was contaminating the phosphorylase, thereby hydrolyzing some of the starch which was being synthesized and thus lowering the results for the phosphorylase activity.

The method used for determining amylase activity was to simply allow some of the enzyme solution to act upon starch in the absence of orthophosphate. Without orthophosphate present, the phosphorylase in the enzyme solution would be dormant, but if amylase was present some of the starch would be hydrolyzed.

The following procedure was used to determine amylase activity present in the phosphorylase solution. A solution containing 1 ml. of 2% starch, 5 ml. veronal buffer (pH 7), 1 ml. of the enzyme solution and 3 ml. of distilled water to bring the mixture to a total volume of 10 ml., was placed in a water bath at 38°C. At intervals of 15 minutes, 1 ml. aliquots were taken and the amount of starch present was determined by the starch-iodine method. The amount of starch hydrolyzed was calculated and plotted against time (Figure 6). The amount of starch hydrolyzed (0.1 mg. / hr.) was negligible compared to the total amount of starch present in the reaction mixture. Therefore, it was safe to consider the phosphorylase solution as being free of amylase.



PHOSPHORYLASE ACTIVITY CURVE

FIGURE 5

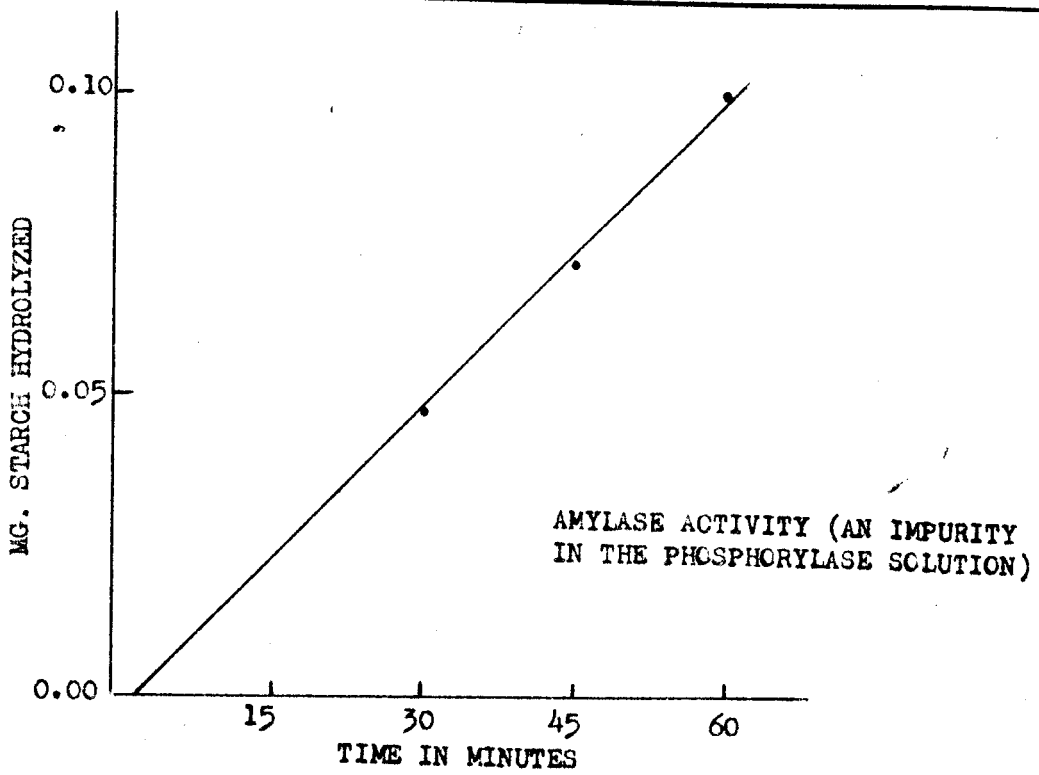


FIGURE 6

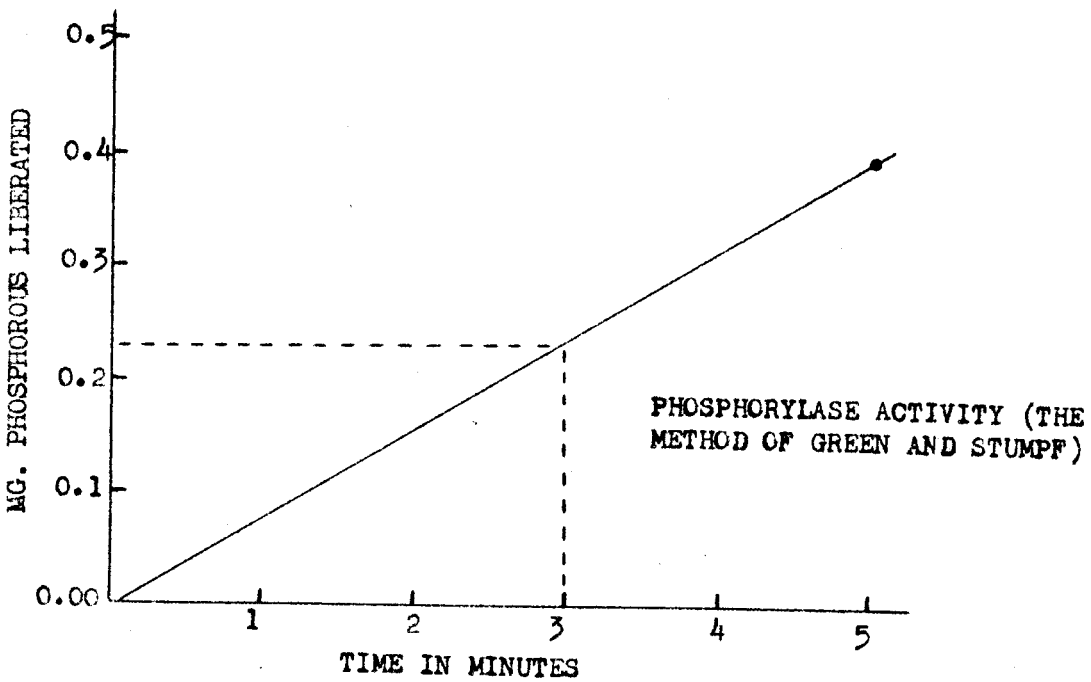


FIGURE 7

Phosphorylase Activity as Determined by the Method of Green and Stumpf (19)

Green and Stumpf (19) defined a unit of phosphorylase activity as the amount of enzyme that catalyzes the liberation of 0.1 mg. of inorganic phosphate from 0.1 millimole glucose-1-phosphate in 3 minutes at 38°C. and pH 6.0.

A 1 ml. aliquot of the phosphorylase solution (prepared as described above) was added to a 25 ml. graduated test tube containing 0.5 ml. veronal buffer pH 6.0 (50), 0.2 ml. of 5% soluble starch (analytical reagent, according to Lintner) and 1 ml. 0.1 M. glucose-1-phosphate (Schwartz Laboratories), the final volume being 3.5 ml. The glucose-1-phosphate was added to the rest of the mixture after temperature equilibration of 38°C. The reaction was stopped after 5 minutes by the addition of 5 ml. of 5% trichloroacetic acid. The solution was diluted with water to the 25 ml. mark and filtered. A 5 ml. aliquot was taken and added to a 50 ml. flask. The determination of liberated orthophosphate was followed according to Sumner (59), that is, 5 ml. of 6.6% ammonium molybdate was added and the solution diluted to approximately 35 ml. with water. Five ml. of 7.5 N. H_2SO_4 was added, then 4 ml. of 10% ferrous sulfate. The solution was diluted to volume and read after 10 minutes in a Junior Coleman Spectrophotometer at 660 mμ. It was found that 1 ml. of phosphorylase liberated 0.394 mg. of phosphorous per 5 minutes which is equivalent to 0.23 mg. of phosphorous per 3 minutes (Figure 7). This amount of liberated phosphorous per 3 minutes is equivalent to 2.3 units of phosphorylase activity as defined by Green and Stumpf (19).

Phosphorylase activity as determined by the amount of starch synthesized was defined in units according to Green and Stumpf (19). For example, 2.3 units of phosphorylase according to Green and Stumpf's method was found to synthesize 12.95 mg. starch per 30 minutes from a solution containing 0.2% glucose-1-phosphate. Therefore, it had been found that 1 unit (Green and Stumpf) of phosphorylase will synthesize 5.63 mg. starch per 30 minutes (starch-iodine method).

The starch-iodine method for determining phosphorylase activity was found to be advantageous over the ordinary methods of determining phosphorylase activity (by determining the amount of phosphate liberated from glucose-1-phosphate) in that only one reagent (0.01 N. iodine) is required and this reagent was found to remain stable over a period of weeks if properly stoppered and stored in a dark place. Also, no specific inactivator is needed to stop the reaction at the end of the incubation period, for the iodine reagent, besides forming the color complex, will also inactivate the enzyme and thus stop the reaction. The starch-iodine color complex forms immediately and is stable for many hours at neutrality and therefore, it is not essential to make the colorimetric readings immediately or within a short period after stopping the reaction.

The Effect of Sodium Monofluorophosphate on Starch Phosphorolysis

A test was first run to determine the concentration of substrate (orthophosphate) which would be needed to react with the starch per unit time to form the glucose-1-phosphate. This was determined by varying the

orthophosphate concentration of the test solution until the activity of the enzyme remained constant with increasing orthophosphate. In this way the concentration of orthophosphate which produced maximum activity of the enzyme could be determined and also the activity of the phosphorylase could be determined as the orthophosphate concentration was varied (Figure 8).

The following method was used to determine the effect of varying concentrations of orthophosphate on phosphorylase activity. To a solution containing 1 ml. 2% starch, 3 ml. veronal buffer pH 7.0, and 1 ml. phosphorylase solution, varying concentrations (0.000 to 0.050 M.) of sodium orthophosphate were added. The enzyme solution was added to the test mixtures after a temperature equilibrium of 38°C. was attained. The activity of the enzyme solution was determined by taking a 1 ml. aliquot of the test solution and determining the amount of starch phosphorolyzed after a period of 30 minutes by the starch-iodine method. It was found that a concentration of 0.02 M. of orthophosphate will cause maximum activity of the enzyme in 30 minutes (Figure 8). In the absence of orthophosphate phosphorylase will not act upon starch. This can clearly be understood by showing an equation for the reaction. Starch + orthophosphate $\xrightleftharpoons{\text{Phosphorylase}}$ glucose-1-phosphate. The equation clearly indicates that glucose-1-phosphate cannot be synthesized from starch without orthophosphate being present.

The effect of sodium monofluorophosphate on phosphorylase, using

TABLE III

THE EFFECT OF SODIUM MONOFLUOROPHOSPHATE
ON
STARCH PHOSPHOROLYSIS BY PHOSPHORYLASE

The reaction contained 1 ml. 2% starch, 3 ml. veronal buffer (pH 7.0), 1 ml. phosphorylase solution, varying amounts of 0.1 M. Na_2PO_3F and water to make a total volume of 10 ml. The activity was measured by the amount of starch phosphorolyzed as determined by the starch-iodine method.

Concentration of Na_2PO_3F	Total Mg. of Starch Phosphorolyzed
0.040	0.30*
0.030	0.00
0.020	0.20*
0.010	0.00
0.005	0.00
0.002	0.00

*Within the limits of experimental error.

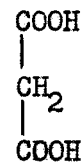
starch phosphorolysis as the means of following the rate of the reaction, was then determined. From the data previously obtained (Figure 8), it was found that phosphorylase will not act upon starch in the absence of orthophosphate. It was also found that phosphorylase will not act upon starch in the presence of monofluorophosphate either (Table III). This effect was determined experimentally by the same method used to determine the orthophosphate concentration as described on page 32, except varying concentrations (0.00 to 0.040 M.) of sodium monofluorophosphate were used in place of sodium orthophosphate. The results of the experiment show that monofluorophosphate cannot replace orthophosphate in the equation, starch + orthophosphate $\underset{\text{Phosphorylase}}{\rightleftharpoons}$ glucose-1-phosphate. From this it can be concluded that a complex, such as glucose-1-monofluorophosphate, is not formed by the action of phosphorylase on starch and monofluorophosphate.

Sodium Monofluorophosphate as a Competitive Inhibitor of Phosphorylase

A great deal of information regarding the nature of enzymes and their mode of action has been gained by considering their inhibition, and studies of this kind have done much to confirm the view that enzymes are made up essentially of protein material. Many enzymes are inhibited by the products of their own activity, and many more by substances which are structurally related to their substrates. In many such cases, the inhibition is of what is known as the competitive type. A well known and very important case is found in the competitive inhibition of succinic dehydrogenase by malonate.

If succinate is taken together with succinic dehydrogenase a system is had in which, under suitable conditions, it is very easy enough to measure the reaction velocity in terms of the rate of oxidation of the succinate. If malonate is added the rate of oxidation promptly diminishes, but increases again if more succinate is added. The malonic acid structure is closely related to that of succinic acid itself.

Succinic Acid



Malonic Acid

Malonate is able to combine with the enzyme, just as does the substrate succinate. But whereas the enzyme-succinate complex breaks down to yield the reaction products, the enzyme-malonate complex contributes nothing to the reaction velocity. In consequence, a part of the enzyme is held in the form of enzyme-inhibitor complex, and so is not available for the catalysis of succinate oxidation, and the reaction velocity accordingly diminishes when the inhibitor is added.

This same phenomenon was found to be true of the inhibition of phosphorylase by monofluorophosphate. The substrate for phosphorylase in the synthesis of glucose-1-phosphate from starch is orthophosphate. It has been found, though, that monofluorophosphate will inhibit this reaction

(Table III). Because of the great similiarity of structures between orthophosphate and monofluorophosphate, it was then thought that possibly monofluorophosphate could be a competitive inhibitor of phosphorylase, competing for the same site of attachment on the enzyme as orthophosphate.

Experimental evidence indicates that monofluorophosphate does act as a competitive inhibitor of phosphorylase. The method used in determining this inhibition was to add sodium monofluorophosphate to a solution containing starch, buffer, and phosphorylase and determine the reaction velocity. Then the orthophosphate concentration was increased and again the reaction velocity determined. The following procedure was used. To each of a series of solutions containing 1 ml. 2% starch, 3 ml. of veronal buffer pH 7.0, 1 ml. 0.1 M. sodium monofluorophosphate and 1 ml. phosphorylase solution, an increasing amount of 0.1 M. sodium orthophosphate solution was added to make the final concentration of the test solutions varying from 0.00 to 0.04 M. with respect to orthophosphate (Table IV). The velocity was expressed in mg. of starch phosphorolyzed per 30 minutes and was determined by the starch-iodine method.

The results indicated that as increasing amounts of orthophosphate were added to the starch-enzyme solution containing 0.01 M. monofluorophosphate, the reaction velocity was increased correspondingly (Figure 8). This indicates that monofluorophosphate is a competitive inhibitor of phosphorylase.

Another means of verifying the competitive inhibition of mono-

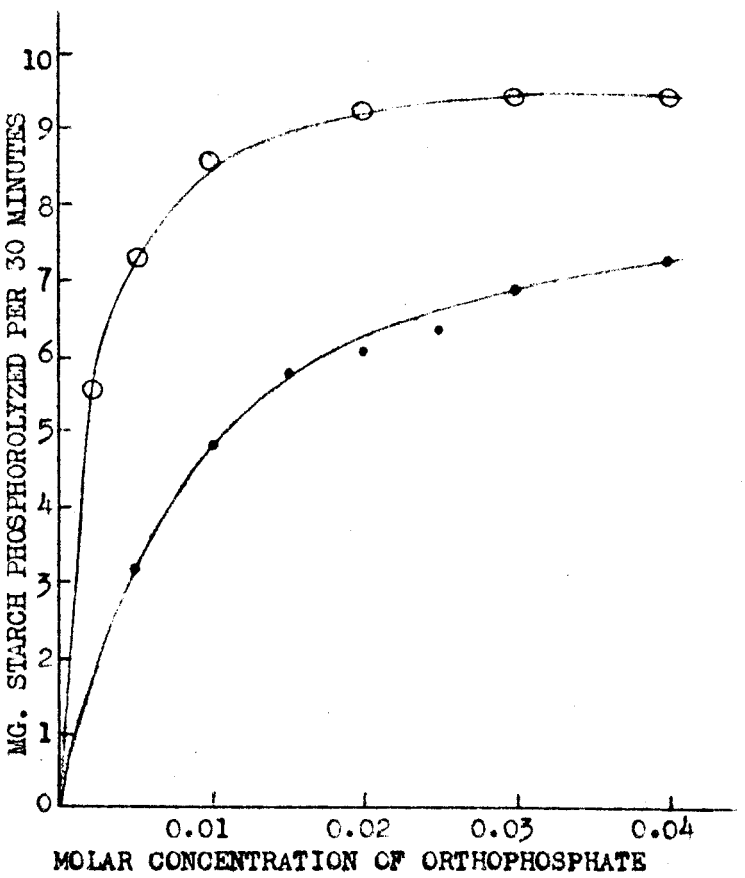
TABLE IV

DATA FOR THE COMPETITIVE INHIBITION OF PHOSPHORYLASE AND THE ORTHOPHOSPHATE CONCENTRATION EFFECT ON PHOSPHORYLASE (FIGURES 8 AND 9)

The reaction mixture contained 1 ml. 2% starch, 3 ml. veronal buffer (pH 7.0), 1 ml. phosphorylase, and 1 ml. 0.1 M. Na_2PO_3F (Na_2PO_3F omitted in mixture B). Varying concentrations of orthophosphate were added to each of the mixtures and the total volume was 10 ml. The mixtures were maintained at 38°C. and the reaction stopped after 30 minutes. Activity was determined by measuring the amount of starch phosphorolyzed by the starch-iodine method.

	Molar Concentration of Phosphate (S)	(1/S)	Mg. Starch Phosphorolyzed (v)*	(1/v x 100)
Mixture A with Na_2PO_3F	0.040	25.0	7.20	13.9
	0.030	33.3	6.80	14.7
	0.025	40.0	6.30	15.9
	0.020	50.0	6.00	16.7
	0.015	66.6	5.70	17.6
	0.010	100.0	4.75	21.1
	0.005	200.0	3.10	32.2
	0.000		0.00	
Mixture B without Na_2PO_3F	0.040	25.0	9.40	10.65
	0.030	33.3	9.40	10.65
	0.020	50.0	9.20	10.87
	0.010	100.0	8.60	11.62
	0.005	200.0	7.30	13.60
	0.002	500.0	5.50	18.10
	0.000		0.00	

*These figures are the average of five runs.



ORTHOPHOSPHATE CONCENTRATION CURVE
AND COMPETITIVE INHIBITION CURVE

ORTHOPHOSPHATE CONCENTRATION CURVE

ORTHOPHOSPHATE CONCENTRATION CURVE IN THE PRESENCE OF MONOFLUOROPHOSPHATE (COMPETITIVE INHIBITION CURVE)

FIGURE 8

fluorophosphate on phosphorylase from the data obtained (Table IV) is by the method of Lineweaver and Burk (40). The method involves plotting the reciprocal of the reaction velocity (v) against the reciprocal of the substrate (orthophosphate) concentration (s) in the presence of the inhibitor (monofluorophosphate) and also in the absence of the inhibitor. If the intercept is unchanged but the slope is increased in the presence of the inhibitor, then the inhibitor is of a competitive nature. This was found to be true with monofluorophosphate as is shown by Figure 9.

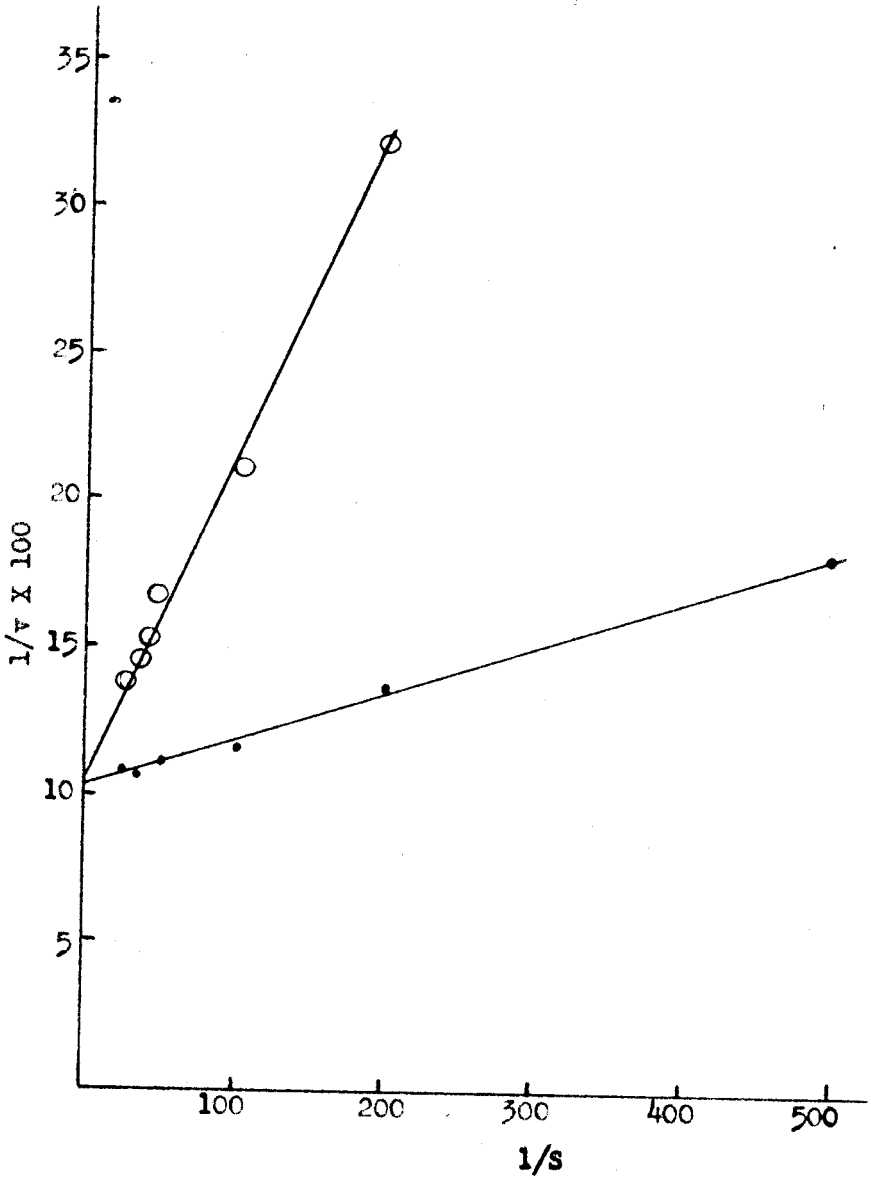
The Effect of Orthophosphate, Monofluorophosphate, and Fluoride on Phosphorylase as Determined by the Synthesis of Starch

It has been shown that monofluorophosphate inhibits the reaction, starch + orthophosphate $\overset{\text{Phosphorylase}}{\rightleftharpoons}$ glucose-1-phosphate. This inhibition was found to be on a competitive basis. The following experiments were devised to show the inhibiting effects of monofluorophosphate on the synthesis of starch from glucose-1-phosphate in the presence of phosphorylase, and thereby checking the conclusions of the previous experiments that monofluorophosphate is an inhibitor of the enzyme phosphorylase. Since orthophosphate now is not a substrate in this reversed reaction but rather a product of the dephosphorylation of glucose-1-phosphate, the effect of orthophosphate on starch synthesis by phosphorylase was studied. In a like manner, the fluoride effect on this reaction was investigated, and along with the effect produced by orthophosphate, was compared to the inhibition of phosphorylase due to monofluorophosphate.

The method followed was basically the same as the method of determining phosphorylase activity by starch synthesis (page 25), except that in this case varying concentrations of monofluorophosphate were added. The same procedure was repeated using varying concentrations of orthophosphate and likewise, the procedure was again repeated using fluoride instead of monofluorophosphate or orthophosphate.

The results indicate that monofluorophosphate, and orthophosphate inhibit the phosphorylase action on the dephosphorylation of glucose-1-phosphate to a rather great extent, whereas fluoride has very slight inhibiting power. Figure 10 is a graph of the activity of the phosphorylase (expressed in mg. of starch synthesized) plotted against the molar concentration of added monofluorophosphate, orthophosphate, and fluoride. Figure 11 is a plot of the per cent inhibition against the molar concentration of added monofluorophosphate, orthophosphate, and fluoride. From this graph it is indicated that a concentration of approximately 0.0021 M. of monofluorophosphate will cause a 50% inhibition of the reaction, whereas a concentration of approximately 0.0042 M. of orthophosphate will cause a 50% inhibition of the reaction, which indicates that monofluorophosphate is about twice as effective an inhibitor of starch synthesis by phosphorylase as is orthophosphate. Fluoride has a very weak effect since a concentration of 0.02 M. will only bring about a 6.4% inhibition.

From the data obtained it was clearly shown that the inhibiting

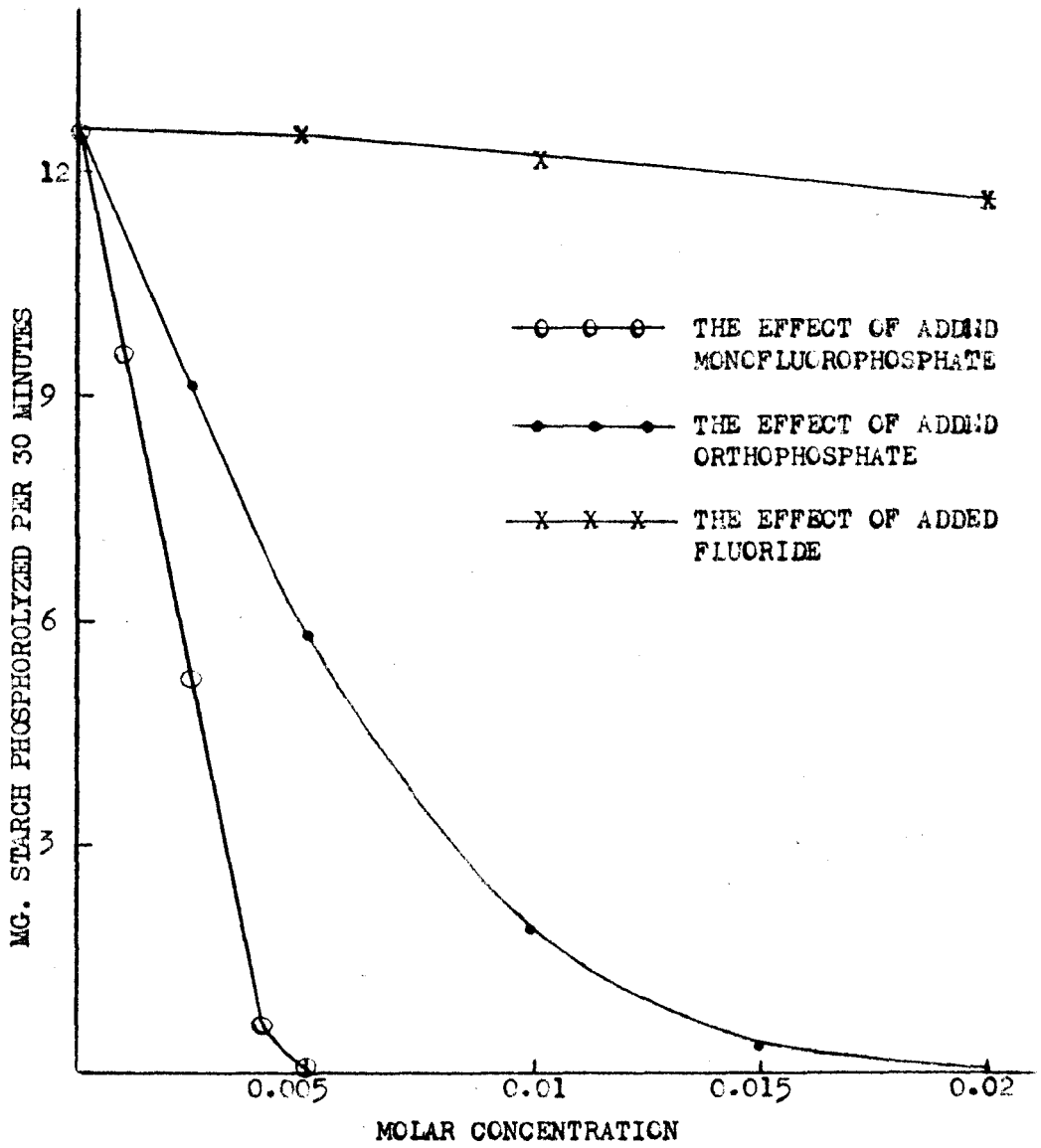


COMPETITIVE INHIBITION COMPUTED
BY THE LINEWEAVER-BURK EQUATION

-o-o-o- MIXTURE WITH MONOFLUOROPHOSPHATE INHIBITOR

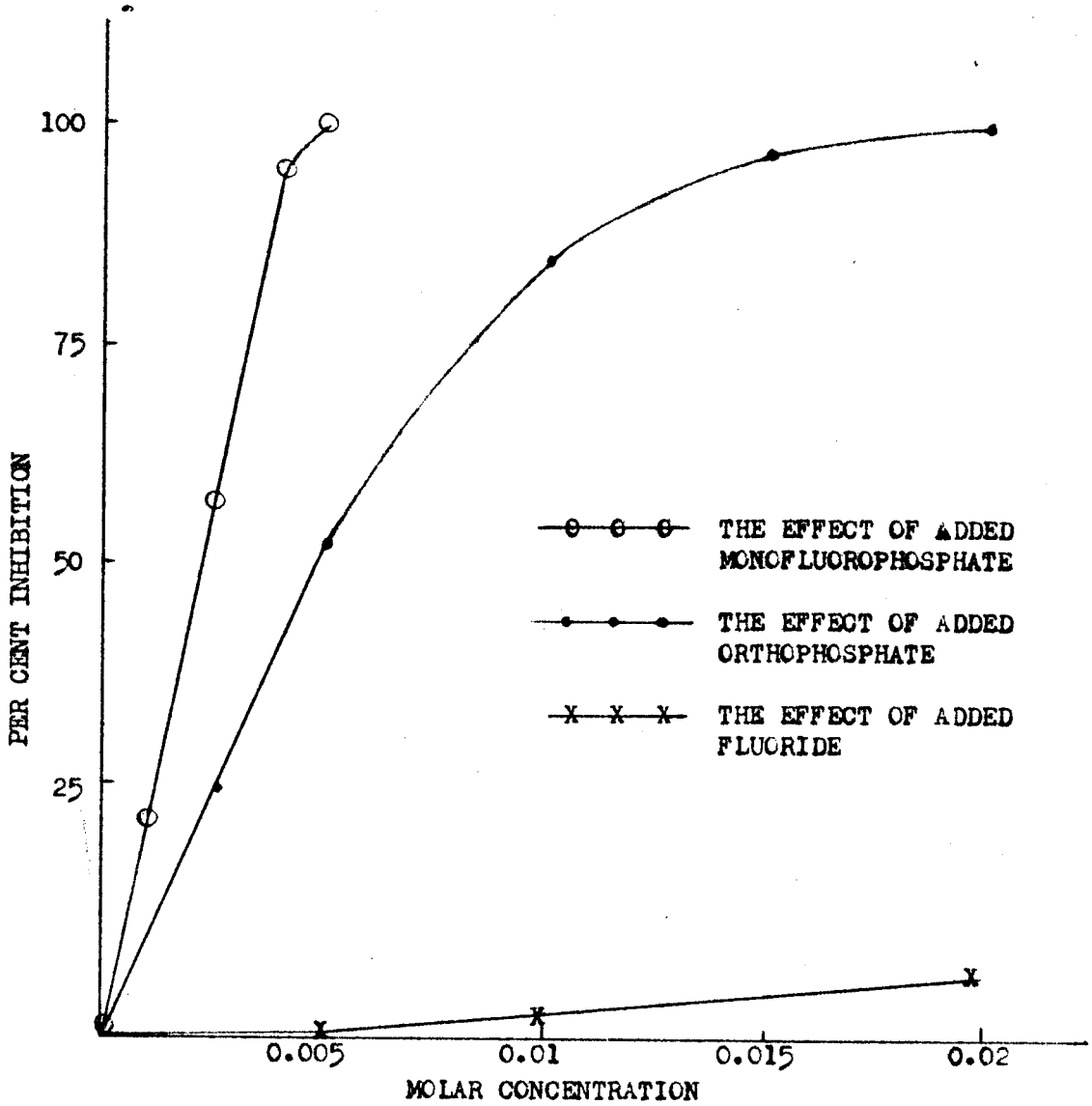
-•-•-•- MIXTURE WITHOUT MONOFLUOROPHOSPHATE INHIBITOR

FIGURE 9



THE EFFECT OF ORTHOPHOSPHATE, MONOFLUOROPHOSPHATE, AND FLUORIDE ON THE SYNTHESIS OF STARCH BY PHOSPHORYLASE

FIGURE 10



THE EFFECT OF ORTHOPHOSPHATE, MONOFLUOROPHOSPHATE, AND FLUORIDE ON THE SYNTHESIS OF STARCH BY PHOSPHORYLASE (PER CENT INHIBITION VERSUS CONCENTRATION)

FIGURE 11

effect produced by monofluorophosphate was not due to the hydrolytic products (fluoride and orthophosphate) of monofluorophosphate but rather to the inhibiting powers of monofluorophosphate itself. If the hydrolytic products of monofluorophosphate caused the inhibition of phosphorylase, then the magnitude of the inhibition due to monofluorophosphate alone would be equivalent to the sum of the inhibitory effects of orthophosphate and fluoride, considering all other conditions to be equal. This was not found to be true as is shown in Figures 10 and 11. Monofluorophosphate has a greater inhibitory effect on phosphorylase than does the sum of equal molar concentrations of orthophosphate and fluoride. From this we can also conclude that the fluoride group of monofluorophosphate is not dissociated from the phosphorous, and therefore the entire monofluorophosphate radical itself is the direct inhibitory agent.

CHAPTER V

ALKALINE PHOSPHATASE

History

Alkaline phosphatase is very widely distributed in nature. It is found in all animal cells with the exception of the hyaline cartilage and the tunics of the vessels as shown by MacFarlane, Patterson, and Robison (42). White blood cells and lymphoid tissues are rich in this enzyme; plant cells contain little (yeast, bacteria) or, more commonly, none (green plants, higher fungi). Its purification has been accomplished by several authors, but the method of Schmidt and Tannhauser (56) seems to work rather well.

The principal properties of alkaline phosphatase are its pH optimum and its sensitivity toward certain modifying factors. The former exhibits small variations depending upon the substrate being studied; it lies between 9.2 and 9.6 for the purified enzymes, but occasionally it is slightly below 9.0 in crude preparations. It can be determined vigorously only during a very short hydrolysis (30 minutes at most) owing to the rapid inactivation of the enzyme in alkaline solution.

The principal activators of alkaline phosphatase are the divalent cations, among them $Mg.^{++}$. Insufficiently purified preparations exhibit a progressive activation by $Mg.^{++}$ in proportion to its concentration up to an optimum level (M/200 in many instances) as shown by Erdtmann (16a,b,c);

beyond that point this effect is less pronounced.

Roche and Nguyen-van-Thoai (54) found that the most important inhibitors of phosphatase are the phosphate and arsenate ions and various metal-complex-forming compounds, such as the cyanides. Martland and Robison (43) indicated that sulfhydryl compounds, particularly cysteine, are powerful inhibitors and the same is true of hydrogen sulfide, while Roche and co-workers (55) found that fluorides and pyrophosphates inhibit to a much lesser degree and under special conditions. Alkaline phosphatase is more stable in neutral or slightly acid solution than at alkaline pH levels and hydrolyzes β-glycerophosphate more rapidly than the α-isomer. Purification may modify the proportion of hydrolysis of the two isomers.

Most of the methods for the determination of phosphatase activity are colorimetric or photometric. In general, it can be stated that the phosphatase solution is allowed to hydrolyze some phosphate in the presence of a suitable buffer at a fixed temperature for a definite period of time. An aliquot of the digest is then analyzed for free orthophosphoric acid. An alternative procedure, which is of value when much inorganic phosphate is already present, consists in employing phenyl-ortho, or pyro-phosphate, or phenolphthalein phosphate as the substrate.

The method of measuring phosphatase activity in the proceeding experiments employed sodium phenolphthalein phosphate as the substrate as introduced by Huggins and Talalay (31). The activity of phosphatase could

be determined colorimetrically by the amount of phenolphthalein liberated from the substrate in a certain time interval. The colorimetric reading (in per cent transmission) was compared to a standard curve so that the activity could be expressed in mgs. of phenolphthalein liberated per unit time, as was described by Huggins and Talalay (31).

Preparation of Sodium Phenolphthalein Phosphate, the Substrate for Phosphatase Determinations

The method used to synthesize sodium phenolphthalein phosphate was as described by Huggins and Talalay (31).

> For 0.1 M. quantities, these directions may be followed. To 32 gm. of phenolphthalein is added fairly rapidly with mechanical stirring a mixture of 50 gm. (30 cc.) of pure, dry phosphorus oxychloride in about 50 cc. of dry chloroform. Dry pyridine, 25 cc., is then added slowly, while stirring rapidly and cooling the reaction mixture with ice. The addition of pyridine should be regulated so that the reaction flask does not become hot. The mixture should be stirred for several hours and should be allowed to stand at least overnight to permit the reaction to go to completion. The degree of completion of the reaction may be determined by withdrawing small samples of the reaction mixture and adding excess alkali. The intensity of the red color is a measure of the amount of free unchanged phenolphthalein and serves as an index of the state of completion of the reaction.
>
> The reaction mixture is evaporated in vacuo at room temperature with slight warming in order to remove most of the chloroform. About 100 cc. of water are then slowly added with stirring and cooling. There is copious evolution of hydrochloric acid, and a white precipitate forms. Strong sodium hydroxide (40 per cent) is added until all the precipitate redissolves and the solution is alkaline to phenolphthalein; the small amounts of phenolphthalein in the solution serve as its own indicator.

The alkaline solution is extracted twice with ether in order to remove pyridine. If the solution is too viscous, some water may be added, although it is desirable to keep the volume as small as possible in order to facilitate the later precipitation of phenolphthalein diphosphoric acid. The aqueous layer is acidified with concentrated hydrochloric acid until blue to Congo red. This precipitates phenolphthalein diphosphoric acid, which is a glistening, viscid mass. When precipitation is approximately complete, the supernatant solution is decanted and the free acid dried in vacuo over calcium chloride. The dry, crude phenolphthalein diphosphoric acid is ground in a mortar to a white powder.

The powdered acid is dissolved in methanol, to which some pyridine has been added to increase the solubility. A solution of sodium ethoxide in ethanol, made by dissolving metallic sodium in absolute ethyl alcohol, is added until no further precipitation occurs. The sodium phenolphthalein phosphate separates as a copious white precipitate which is filtered off or the supernatant decanted. The precipitate is washed with repeated changes of alcohol and ether, until it is quite dry.

The preparation at this stage should be white and give almost no color in alkaline solution. It is repurified by dissolving in a mixture of methanol (80 parts by volume) and formamide (20 parts by volume), reprecipitating with absolute ethanol, and washing the precipitate with ethanol and ether. Reprecipitation from aqueous solution is not so satisfactory, since the sodium salt comes down as a sticky mass. It has not been possible to crystallize this compound in good yield; none of the preparations has contained free phenolphthalein, although inorganic phosphate is usually present in small amount. The sodium phenolphthalein phosphate should be kept dry, dark, and cold. Under these conditions it remains quite stable.

Standard Phenolphthalein Calibration Curve

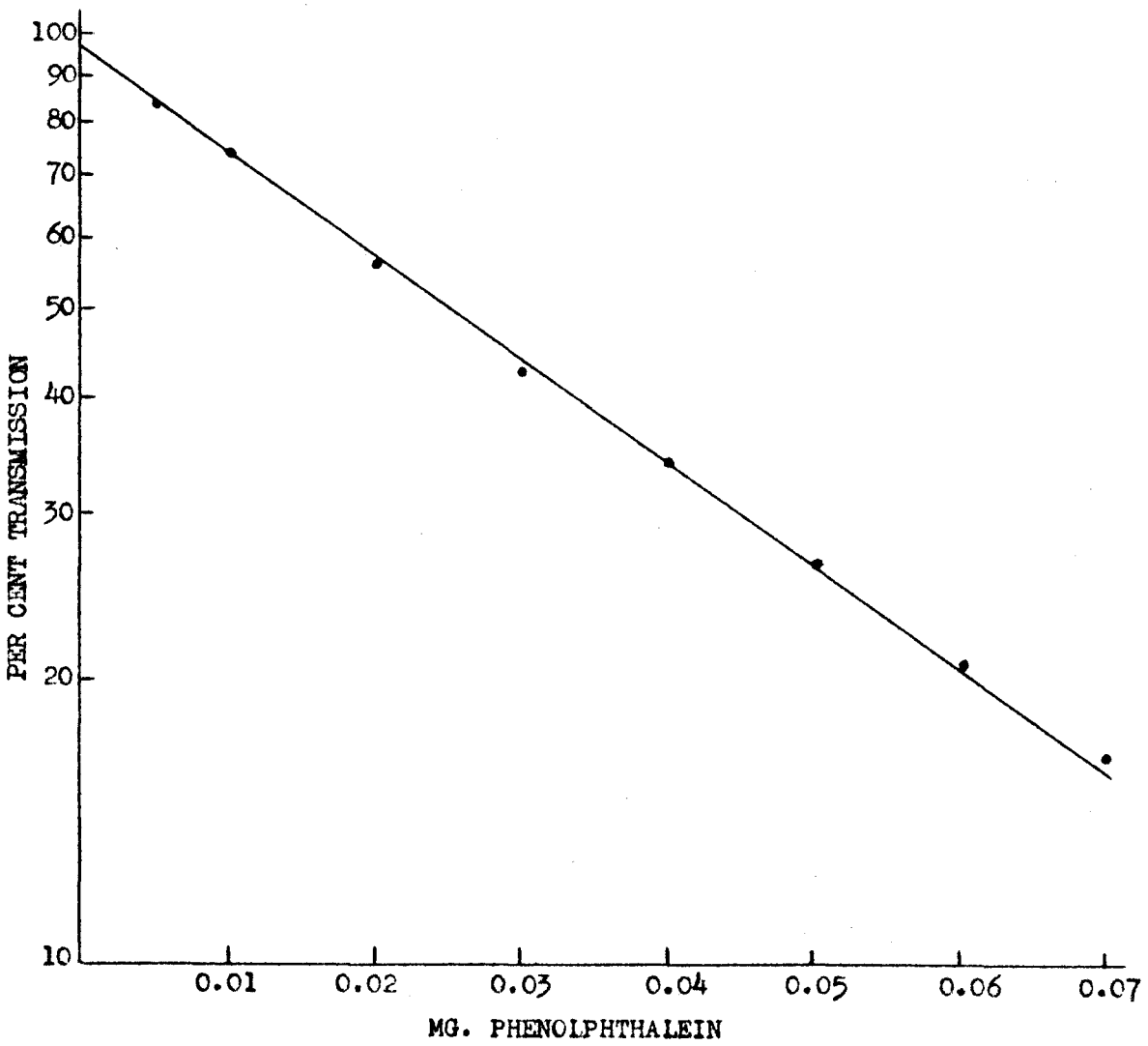
The standard phenolphthalein calibration curve was determined by using a stock phenolphthalein solution. The test was performed in well

matched colorimeter tubes which were washed until they were scrupulously free from acids or alkalies used in cleaning. The rubber stoppers are boiled and freed from "bloom," since this contaminant has acted as a phosphatase inhibitor. A Junior Coleman Spectrophotometer set at a wave length of 540 mμ. was used throughout.

Dilutions of stock phenolphthalein solution were made, i.e. 1, 2, 4, 6, 8, 10, 12, and 14 ml. of stock phenolphthalein per liter of distilled water. Five ml. of each dilution were placed in a colorimeter tube and 5 ml. of glycine buffer for color development for phosphatase tests. The color density was determined immediately after mixing in a colorimeter. The per cent transmission was plotted against mg. of phenolphthalein on semi-log paper and the points fall on a straight line (Figure 12).

The alkaline buffered substrate of phenolphthalein phosphate used throughout all the tests with phosphatase was prepared as follows: mix 20.6 gm. of sodium barbital and 0.608 gm. of sodium phenolphthalein phosphate in distilled water and dilute to 1 liter. Add 7.5 ml. chloroform. The pH of the solution is 9.7. This solution contains 0.001 M. of sodium phenolphthalein phosphate.

In all quantitative phosphatase tests, it is assumed that the amount of product formed under standard conditions is proportional to the enzyme concentration, provided an excess of substrate is used. This was confirmed for orthophosphate liberated from sodium β-glycerophosphate and



STANDARD PHENOLPHTHALEIN CALIBRATION CURVE

FIGURE 12

for phenol liberated from disodium monophenylphosphate, in each case with a wide range of enzyme concentrations. In the case of the enzymic hydrolysis of sodium phenolphthalein phosphate, the relation between the amount of orthophosphate liberated and enzyme concentration is entirely rectilinear, but the relation between phenolphthalein liberated as determined colorimetrically and the enzyme concentration is a parabolic curve (31).

The reason for this discrepancy between the amount of phosphorous liberated and the amount of phenolphthalein as determined colorimetrically is not known. It is probably due to the existence of two hydrolytic products of phenolphthalein diphosphate.

Analysis of the curve of phenolphthalein liberated versus enzyme concentration, revealed that it is a strict parabola which can be resolved into a linear graph by a plot of the log of liberated phenolphthalein against the log of the enzyme concentration.

The enzyme phosphatase which was used throughout all the following experiments involving phosphatase was purchased from Delta Chemical Works, New York City, New York, and was prepared by the method of Schmidt and Thannhauser (56).

A time curve was run using the alkaline phenolphthalein phosphate substrate to determine at what convenient time interval it would be best to stop the reaction. The following procedure was used. To a solution containing 10 ml. of 0.001 M. alkaline substrate (sodium phenolphthalein

phosphate); was added 1.0 ml. of phosphatase solution (containing 1 mg. of purified phosphatase per ml.). Water was added to bring the total volume to 20 ml. At time intervals of 5, 10, 15, 30, 45, and 60 minutes, the reaction was stopped by taking a 5 ml. aliquot and adding it to a 50 ml. volumetric flask containing 5 ml. of glycine buffer. The glycine buffer which contains pyrophosphate inactivates the enzyme so that by adding the test mixture to the glycine buffer solution the reaction is stopped immediately.

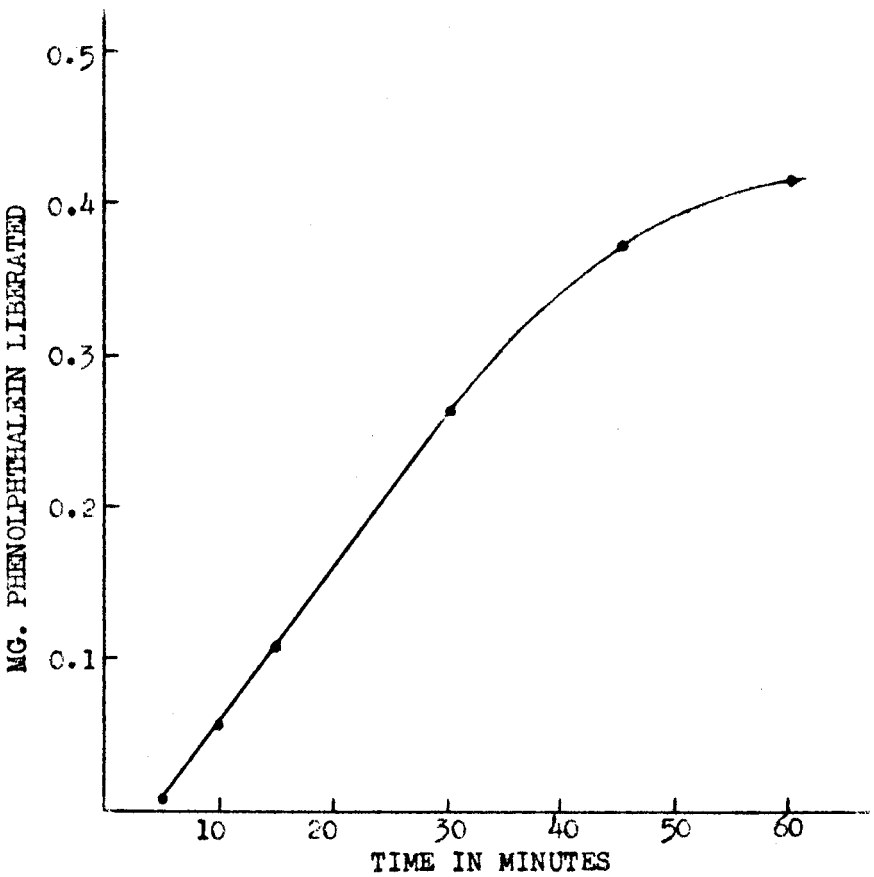
Figure 13 is a plot of time versus the activity of phosphatase expressed as mg. of phenolphthalein liberated per unit time. The graph shows that up to a period of 30 minutes, the amount of phenolphthalein liberated is proportional with time and the plot is strictly linear. Therefore, an incubation period of 30 minutes was used throughout all the tests.

A series of tests was carried out to determine the effect of varying concentrations of phosphatase on the rate of the reaction. A series of tubes (graduated to 20 ml.) containing 10 ml. sodium phenolphthalein phosphate substrate, enzyme concentrations ranging from 0.5 to 2.0 mg. and water to make a total volume of 20 ml., was placed in a water bath for 30 minutes at 38°C. The phenolphthalein liberated was determined by the method previously described. That is, a 5 ml. aliquot was placed in a 50 ml. volumetric containing 5 ml. of glycine buffer and the solution was diluted to volume. The colorimetric readings were for

the 5 ml. aliquot. To determine the total mg. of phenolphthalein liberated, the amount of phenolphthalein liberated per aliquot was multiplied by a factor of 10. The amount of phenolphthalein liberated with increasing enzyme concentrations was found to be a linear plot if the log of the per cent transmission was plotted against the log of the enzyme concentration. This plot was made by using the log-log type of graph paper (Figure 14).

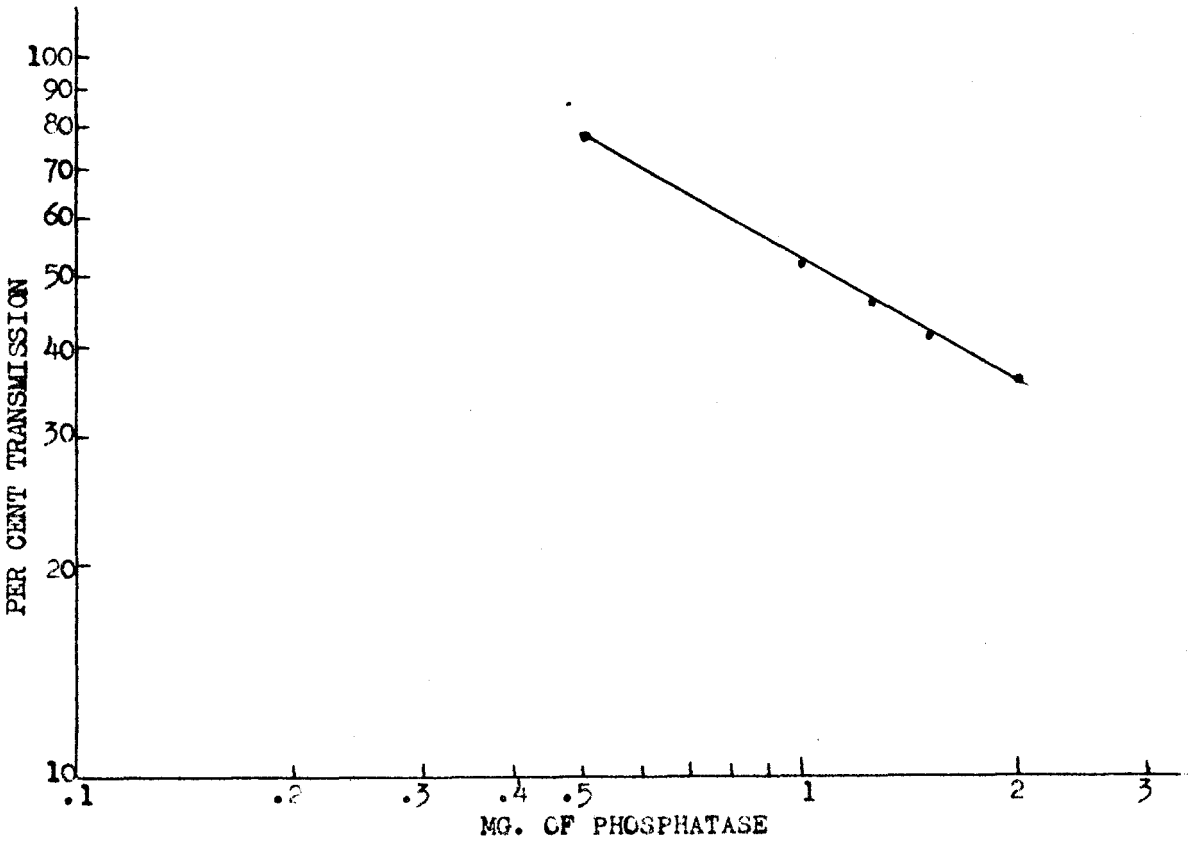
The Effect of Orthophosphate, Monofluorophosphate and Fluoride on Phosphatase

The effect of orthophosphate and monofluorophosphate have been determined on the enzymic phosphorylation of starch and dephosphorylation of glucose-1-phosphate by phosphorylase. It seemed to be of importance to determine what effect orthophosphate and monofluorophosphate along with fluoride would have on the enzyme phosphatase. Since it has been shown that orthophosphate and monofluorophosphate does inhibit the reaction, glucose-1-phosphate $\underset{\leftarrow}{\overset{\text{Phosphorylase}}{\rightleftharpoons}}$ starch + orthophosphate, where orthophosphate is split from the ester, it would seem that orthophosphate and monofluorophosphate would have the same effect on phosphatase since it has essentially the same action of splitting phosphate from the phosphate esters. Experiments to show the effect of orthophosphate and monofluorophosphate on phosphatase were carried out and compared to the effect which fluoride has on phosphatase. This comparison was made to determine whether monofluorophosphate itself is the inhibitor or whether it is the



THE ACTIVITY OF PHOSPHATASE
PLOTTED AGAINST TIME

FIGURE 13



LOG-LOG PLOT OF PHENOLPHTHALEIN LIBERATED EXPRESSED AS PER CENT TRANSMISSION VERSUS PHOSPHATASE CONCENTRATION

FIGURE 14

combined effects of the fluoride and orthophosphate, formed from the hydrolysis of monofluorophosphate in the reaction mixture, which is causing the inhibition.

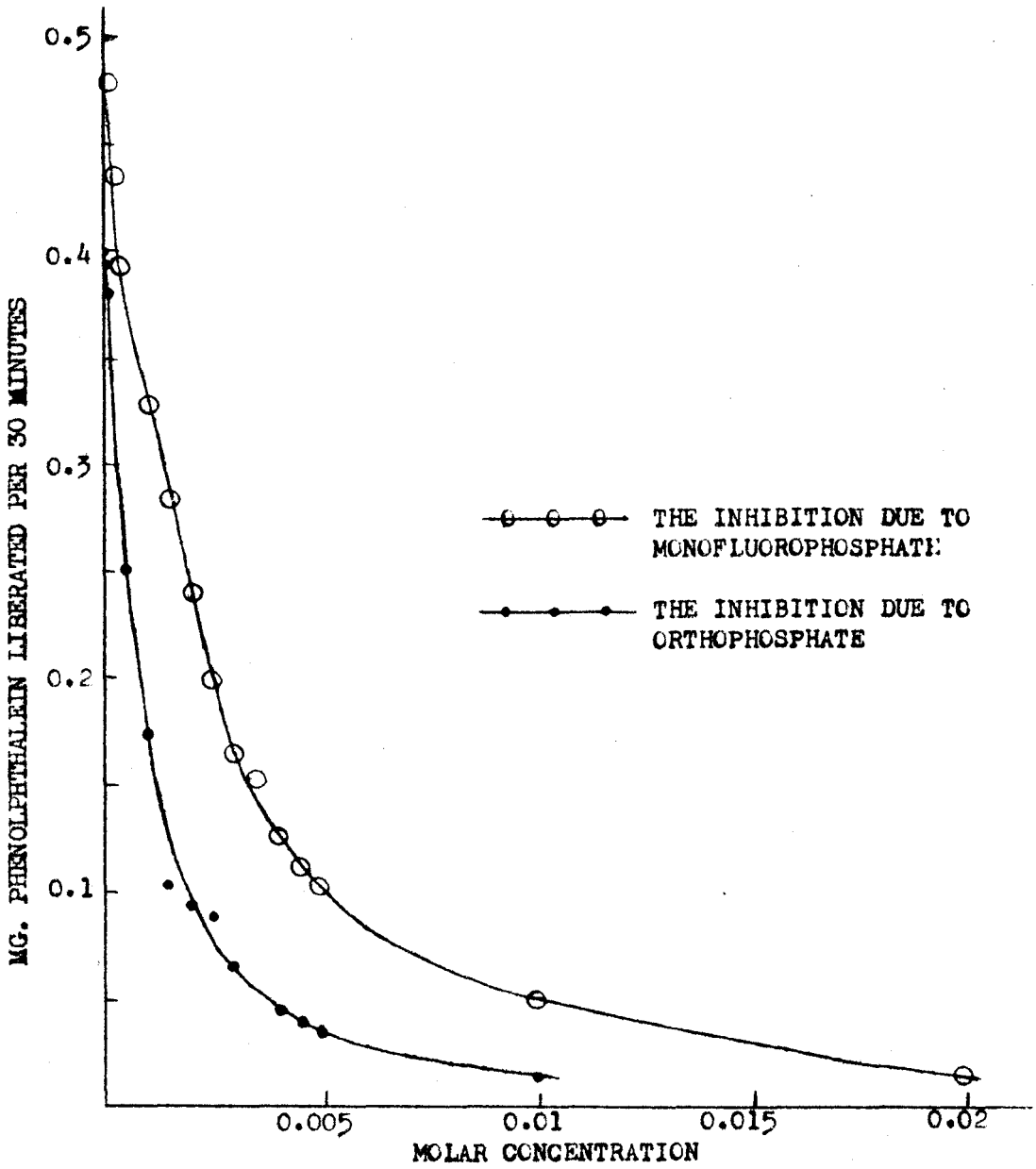
The following procedure was applied to the tests on monofluorophosphate. To a series of graduated test tubes was added 10 ml. 0.001 M. alkaline sodium phenolphthalein phosphate solution and 1 ml. of phosphatase solution (containing 1 mg. of phosphatase per ml.). Then sodium orthophosphate (monohydrogen) solution was added and water to make a total volume of 20 ml. The total concentration of sodium orthophosphate was in a range of 5×10^{-5} to 1×10^{-2} M. The reaction mixture was allowed to reach a temperature equilibrium of 38°C. before the phosphatase was added. After a time interval of 30 minutes the reaction was stopped by placing a 5 ml. aliquot in a 50 ml. volumetric flask containing 5 ml. of glycine buffer. The flask was diluted to volume and a portion placed in colorimeter tubes and read at a wave length of 540 mμ. in the Junior Coleman Spectrophotometer (Model 6B). By diluting to a volume of 50 ml. a dilution factor of 10 is involved so that the amount of phenolphthalein which is determined by the standard chart is multiplied by a factor of 10 to give the total mg. of phenolphthalein liberated.

Figure 15 shows the orthophosphate concentration plotted against mg. of phenolphthalein liberated per 30 minutes. Figure 16 shows the orthophosphate concentration plotted versus per cent inhibition. From these curves it can be said that phosphate does inhibit phosphatase

activity and that the very low concentrations produce a greater degree of change in phosphatase activity relative to the amount of orthophosphate added as compared with the activity when higher concentrations of orthophosphate were added. The concentration of orthophosphate is not a linear function of activity or of inhibition.

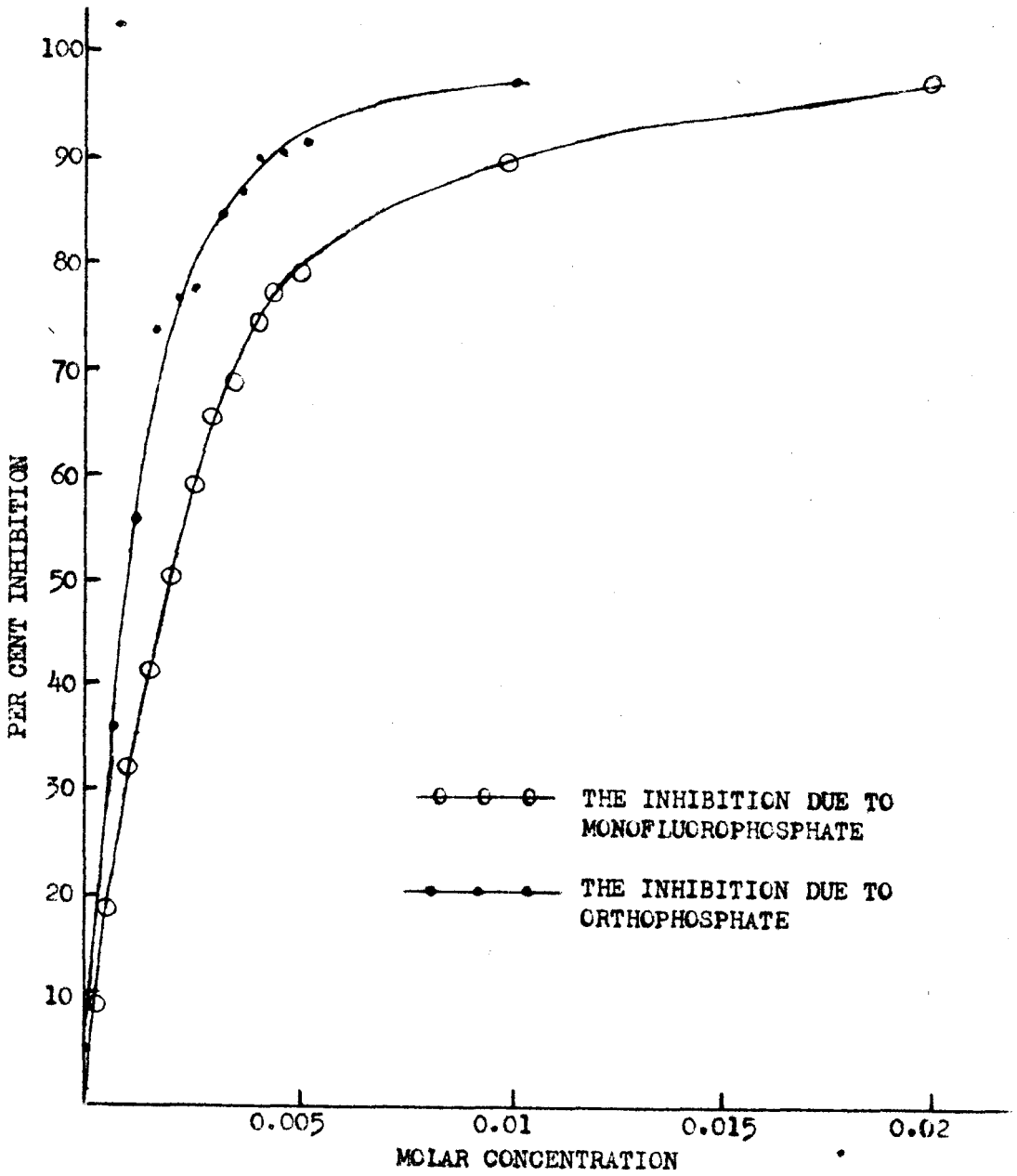
The same procedure as was used in determining the effect of orthophosphate on phosphatase was followed for determining the effect of monofluorophosphate on phosphatase activity. Figure 15 is a plot showing the effect of monofluorophosphate on phosphatase activity, plotted as the monofluorophosphate concentration versus activity expressed in mg. of phenolphthalein liberated. Figure 16 is a plot of the monofluorophosphate concentration versus the per cent inhibition. As with orthophosphate, monofluorophosphate does inhibit phosphatase activity, and likewise, the concentration of monofluorophosphate is not a linear function of activity or of inhibition. Therefore, the very low concentrations of monofluorophosphate produce a greater degree of change in phosphatase activity than do the higher concentrations. It was also noted that orthophosphate has a greater effect on inhibiting phosphatase than does monofluorophosphate. An approximately 0.0009 molar concentration of orthophosphate causes a 50% inhibition of phosphatase whereas a 0.002 molar concentration of monofluorophosphate causes a 50% inhibition.

The effect of sodium fluoride on phosphatase activity was determined in order to compare with the effects produced by orthophosphate



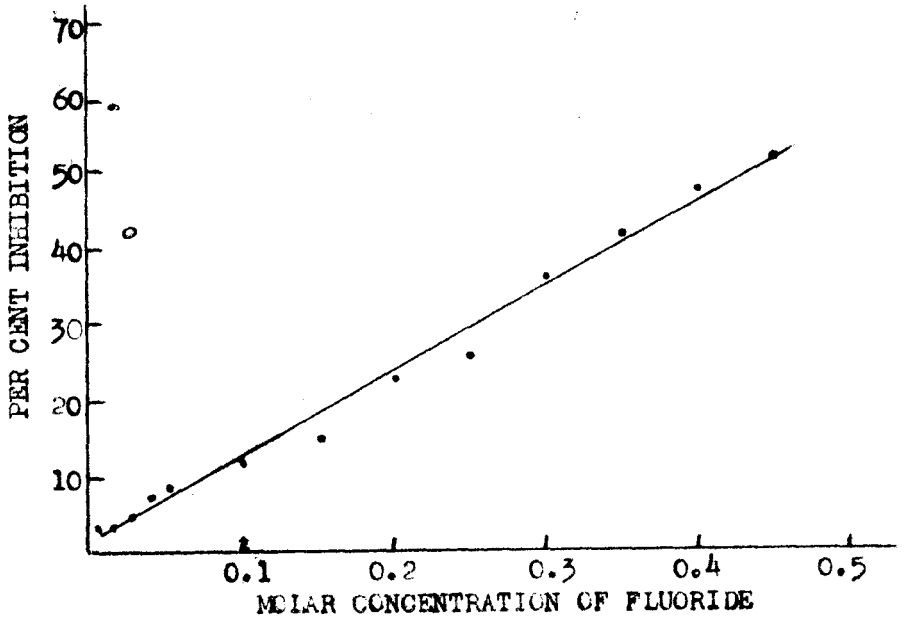
THE INHIBITION CURVE (ACTIVITY VERSUS CONCENTRATION) OF PHOSPHATASE BY ORTHOPHOSPHATE AND MONOFLUOROPHOSPHATE

FIGURE 15



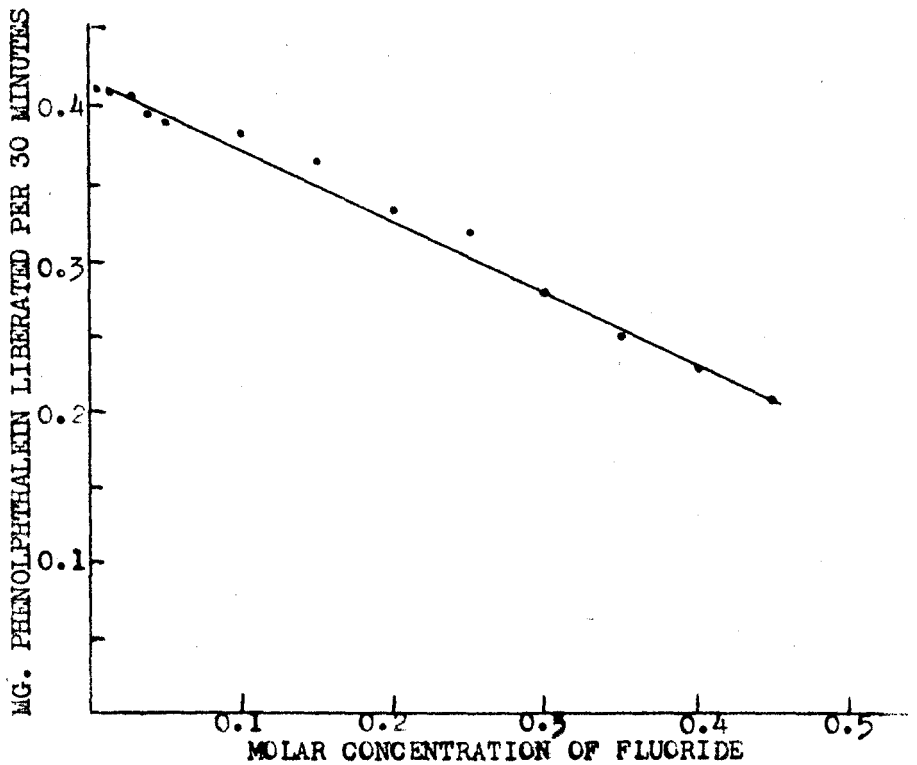
THE INHIBITION CURVE (PER CENT INHIBITION VERSUS CONCENTRATION) OF PHOSPHATASE BY ORTHOPHOSPHATE AND MONOFLUOROPHOSPHATE

FIGURE 16



THE EFFECT OF FLUORIDE ON PHOSPHATASE ACTIVITY
(PER CENT INHIBITION VERSUS CONCENTRATION)

FIGURE 17



THE EFFECT OF FLUORIDE ON PHOSPHATASE ACTIVITY
(MG. OF PHENOLPHTHALEIN LIBERATED VERSUS CONCENTRATION)
FIGURE 18

and monofluorophosphate, thereby, determining whether the inhibition produced by monofluorophosphate was of its own accord, or whether it was due to the sum of the inhibitions due to orthophosphate plus fluoride, the hydrolytic products of monofluorophosphate.

The procedure followed was the same as used in determining the orthophosphate and monofluorophosphate effects on phosphatase. Figure 18 is a plot of the concentration of fluoride versus the activity expressed in mg. of phenolphthalein liberated per 30 minutes. Figure 17 is a plot of the concentration of fluoride versus the per cent inhibition produced by fluoride on phosphatase. Fluoride was found to be a relatively weak inhibitor of phosphatase as compared to orthophosphate and monofluorophosphate. It was determined that a concentration of approximately 0.438 M. produces only 50% inhibition.

Therefore it can be concluded that monofluorophosphate exerts its own type of inhibition and it is not the combined effect of its breakdown products, orthophosphate and fluoride.

CHAPTER VI

CONCLUSIONS

The primary purpose of this investigation was to determine the effects of sodium monofluorophosphate on the activity of the enzymes, phosphorylase and phosphatase. In the process of determining these effects, it was found that sodium monofluorophosphate can be precipitated out with barium acetate in the same manner by which sodium phosphate is precipitated out almost quantitatively with barium acetate. Thus it appears that possibly a complex of barium monofluorophosphate is formed which is a white precipitate similar to barium phosphate and is also insoluble in aqueous solution.

The effect of sodium monofluorophosphate on phosphorylase in the synthesis of glucose-1-phosphate was determined. It was found that monofluorophosphate cannot replace orthophosphate in the reaction, starch + orthophosphate $\underset{\longleftarrow}{\overset{\text{Phosphorylase}}{\longrightarrow}}$ glucose-1-phosphate. From this it was concluded that even though monofluorophosphate might act like orthophosphate in some cases (such as being able to be precipitated out with barium) it does not act like orthophosphate when the requirements become more specialized, such as forming a complex of glucose-1-monofluorophosphate by phosphorylase. Another example of monofluorophosphate being unlike orthophosphate is that it will not form a colored complex with molybdate as does orthophosphate.

Monofluorophosphate was found to be a competitive inhibitor of phosphorylase in the synthesis of glucose-1-phosphate. The reason for this type of inhibition can be clearly seen if the structure of orthophosphate is compared to the structure of monofluorophosphate. Because of the close similarity of the structures it is not too difficult to understand why monofluorophosphate does inhibit competitively. The monofluorophosphate can be said to be able to combine with phosphorylase at the very sites and in the same manner that orthophosphate attaches itself to the enzyme. But, where the phosphorylase-phosphate complex breaks down to form glucose-1-phosphate, the phosphorylase-monofluorophosphate contributes nothing to the reaction velocity.

The inhibition of phosphorylase by monofluorophosphate was checked by determining the effect on the reverse reaction, that is, the dephosphorylation of glucose-1-phosphate by the enzyme. The phosphorylase activity was followed by determining by the starch-iodine method the amount of starch synthesized from glucose-1-phosphate. Orthophosphate and fluoride inhibit phosphorylase also. On the basis of using 50% inhibition as a means of comparison, it was found that 0.0021 M. of monofluorophosphate will cause a 50% inhibition whereas a concentration of approximately 0.0042 M. of orthophosphate will cause the same degree of inhibition. A 0.02 M. concentration of fluoride will inhibit the synthesis of starch by phosphorylase to the extent of only 6.4%. On this basis then, it would be safe to state that orthophosphate inhibits the reaction

of the formation of starch and orthophosphate from glucose-1-phosphate by phosphorylase, because the added orthophosphate attaches itself to that site on the enzyme where the orthophosphate from the glucose-1-phosphate is usually attached. Therefore the glucose-1-phosphate is prevented from forming the phosphorylase-glucose-1-phosphate complex because orthophosphate has occupied one of the attachment points of the glucose-1-phosphate, and formed a phosphorylase-phosphate complex instead.

This same phenomenon occurs when monofluorophosphate is present in the enzyme mixture, but in the case of monofluorophosphate it is thought that the complex formed (phosphorylase-monofluorophosphate) is a more firmly bound complex than is the phosphorylase-phosphate complex and because of this stronger binding, the monofluorophosphate exhibits greater inhibiting powers.

It can also be concluded from the experimental data that the inhibiting power of monofluorophosphate is not due to the combination of the inhibiting power of orthophosphate plus fluoride. It might be thought that the monofluorophosphate might either be completely or partially hydrolyzed in the enzyme solution and that it is the combined inhibiting effects of the hydrolysis products (orthophosphate and fluoride) of monofluorophosphate that causes the inhibition of the reaction whereby glucose-1-phosphate is dephosphorylated by phosphorylase. Experimental evidence shows that this is not the means by which monofluorophosphate exerts its inhibiting effects because the inhibition due to equal molar quantities of

orthophosphate and fluoride would not be as great as the inhibition due to the same concentration of monofluorophosphate alone as indicated in Figures 10 and 11.

Orthophosphate, monofluorophosphate and fluoride were found to have a slightly different effect on the enzyme phosphatase as compared to phosphorylase. With phosphatase, 0.0009 M. of orthophosphate causes a 50% inhibition, whereas 0.002 M. of monofluorophosphate will produce the same degree of inhibition. Fluoride causes a 50% inhibition at a concentration of 0.438 M. Unlike the action of orthophosphate and monofluorophosphate on phosphorylase, it was found that orthophosphate is approximately twice as effective an inhibitor of phosphatase as is monofluorophosphate. The reason for this opposite phenomenon is not clearly understood and at present cannot be explained. It could be thought though, that the nature of these enzymes might possibly afford some means of explaining this phenomenon. The fact that phosphorylase is a specific enzyme for the reversible reaction of orthophosphate on starch or glycogen and that phosphatase is a non-specific enzyme which splits the phosphate radical from a great number of phosphate esters, is not in itself an explanation, but it may lead the way to ideas which might explain this phenomenon.

CHAPTER VII

SUMMARY

1. Orthophosphate could not be determined accurately when present with monofluorophosphate because the monofluorophosphate hydrolyzed at a fairly rapid rate in the acid medium in which orthophosphate is determined, and reproduceable results cannot be obtained.

2. Sodium monofluorophosphate can be precipitated with barium acetate in the same manner that barium acetate precipitates sodium orthophosphate.

3. A method for determining phosphorylase activity by the amount of starch synthesized from glucose-1-phosphate is described. When phosphorylase is expressed in units as compared to a unit of phosphorylase as defined by Green and Stumpf (19), it was found that 1 unit of phosphorylase will synthesize 5.63 mg. of starch in 30 minutes. This starch-iodine method was found to be advantageous over the ordinary methods of determining phosphorylase activity.

4. The effect of sodium monofluorophosphate on the synthesis of glucose-1-phosphate by phosphorylase was described. It was found that monofluorophosphate, unlike orthophosphate, will not enter into the reaction of synthesizing glucose-1-phosphate

from starch. Thus, it can be concluded that the complex glucose-1-monofluorophosphate is not formed by the action of phosphorylase on starch and monofluorophosphate.

5. Sodium monofluorophosphate was found to inhibit phosphorylase competitively in the reaction of synthesizing glucose-1-phosphate from starch and orthophosphate. The method for determining the competitive inhibition was described.

6. Orthophosphate, monofluorophosphate and fluoride were found to inhibit the synthesis of starch by phosphorylase, but in different degrees. In the method described, a concentration of 0.0021 M. of monofluorophosphate will cause a 50% inhibition of the reaction, and a concentration of 0.0042 M. of orthophosphate will cause a 50% inhibition, whereas a concentration of 0.02 M. of fluoride will only bring about a 6.4% inhibition. Thus, monofluorophosphate is approximately twice as effective an inhibitor, under the conditions mentioned, as is orthophosphate.

7. Orthophosphate, monofluorophosphate, and fluoride also inhibit phosphatase in liberating phenolphthalein from the sodium phenolphthalein phosphate substrate. Unlike the action of orthophosphate and monofluorophosphate on phosphorylase, it was found that orthophosphate is a more

effective inhibitor of the reaction on phenolphthalein phosphate than is monofluorophosphate. A concentration of approximately 0.0009 M. of orthophosphate will cause a 50% inhibition whereas a concentration of 0.002 M. of monofluorophosphate causes a 50% inhibition. Fluoride was found to cause a 50% inhibition at a concentration of approximately 0.438 M.

APPENDIX

ORTHOPHOSPHATE DETERMINATION BY THE METHOD OF SUMNER (59)

Reagents:

1. Standard potassium dihydrogen phosphate. 1 ml. is equivalent to 0.5 mg. of phosphorous.
2. Ammonium molybdate. (analytical reagent) 6.6 per cent.
3. Sulfuric acid. 7.5 N. (analytical reagent).
4. Ferrous sulfate. 10 per cent (analytical reagent). This is made up fresh each day with 2 ml. of 7.5 N. sulfuric acid added per 100 ml. solution.

Method for the Determination of Orthophosphate:

a. Put orthophosphate sample in a 100 ml. flask.
b. Add 10 ml. 6.6% ammonium molybdate.
c. Add distilled water to about 75 ml.
d. Add 10 ml. 7.5 N. sulfuric acid.
e. Add 8 ml. 10% ferrous sulfate.
f. Dilute to 100 ml. mark and allow to stand 10 minutes for color to develop. Read versus reagent blank at a wave length of 660 mμ.

TABLE V

STANDARD CURVE DATA FOR ORTHOPHOSPHATE DETERMINATION

(FIGURE 2)

Tube No.	Mg. Phosphorous	Per cent Transmission		
1	0.05	83.5	83.50	84.00
2	0.10	70.5	70.50	70.25
3	0.15	60.0	59.25	59.50
4	0.20	50.8	50.00	50.00
5	0.25	43.0	42.50	42.25
6	0.30	36.5	36.00	36.20
7	0.35	31.0	30.80	30.50
8	0.40	26.0	26.50	26.00
9	0.45	22.0	22.20	23.00
10	0.50	18.5	19.00	18.80
11	0.55	15.8	16.00	16.00
12	0.60	13.5	14.00	14.20
13	0.65	11.3	12.20	11.80

STANDARD STARCH-IODINE CURVE

Reagents:

1. Standard starch solution. 2% soluble starch (Bakers Analyzed Analytical Reagent, according to Lintner). 2 gm. of starch were placed in a 100 ml. flask. About 20 ml. of water were added to suspend the starch. Approximately 70 ml. of boiling water were added rapidly into the starch suspension. On cooling, water was added to volume. This procedure yields 100 ml. of a 2% starch solution which is dissolved and almost water clear.

2. Iodine reagent. A stock solution of 0.1 N. iodine containing 3% potassium iodide was made and standardized against a standard sodium thiosulfate solution. The final iodine solution (0.01 N.) was made by diluting 100 ml. of the stock solution to 1000 ml. The iodine reagents are stored in a cool, dark place.

TABLE VI

STANDARD STARCH-IODINE CURVE DATA

(FIGURE 4)

Into each of a series of 100 ml. flasks containing 4 ml. of the 0.01 N. iodine reagent was delivered progressive amounts of starch from 0.1 to 5.6 mg. Each flask was diluted to the mark and a sample of the contents was read in the Junior Coleman Spectrophotometer in standard colorimeter tubes with a wave length setting of 660 mμ.

Ml. of 0.2% Starch Solution Containing 2.0 mg/ml.	Mg. Starch	% Transmission*
Water blank	0.0	100.00
Iodine blank	0.0	98.00
0.1	0.2	91.25
0.2	0.4	85.00
0.3	0.6	79.50
0.4	0.8	74.50
0.5	1.0	69.50
0.6	1.2	65.00
0.7	1.4	60.50
0.8	1.6	56.80
0.9	1.8	53.00
1.0	2.0	49.00
1.1	2.2	46.00
1.2	2.4	43.00
1.3	2.6	40.50
1.4	2.8	37.00
1.5	3.0	35.00
1.6	3.2	33.25
1.7	3.4	31.00
1.8	3.6	29.00
1.9	3.8	27.00
2.0	4.0	25.50
2.1	4.2	24.00
2.2	4.4	22.00
2.3	4.6	21.00
2.4	4.8	20.00

TABLE VI

(continued)

Ml. of 0.2% Starch Solution Containing 2.0 mg/ml.	Mg. Starch	% Transmission*
2.5	5.0	18.75
2.6	5.2	17.50
2.7	5.4	16.50
2.8	5.6	15.50

*These figures are the average of four runs.

TABLE VII

PHOSPHORYLASE ACTIVITY DETERMINED BY STARCH SYNTHESIS

(FIGURE 5)

The test solutions consisted of 4 ml. of veronal buffer pH 7.0, 2.0 ml. 1% glucose-1-phosphate, 0.1 ml. 5% starch solution, 1 ml. phosphorylase solution, and 2.9 ml. distilled water to bring the total volume to 10 ml. The amount of starch formed was determined by the starch-iodine method.

Test Solution	Time Interval in Minutes	Total mg. of Starch Formed
1	0	0.0
	15	6.6
	30	12.9
	45	19.2
	60	25.9
2	0	0.0
	15	6.5
	30	13.4
	45	19.6
	60	26.4
3	0	0.0
	15	6.8
	30	12.4
	45	19.0
	60	25.7

TABLE VIII

THE DETERMINATION OF AMYLASE PRESENT AS AN IMPURITY IN THE PHOSPHORYLASE SOLUTION

(FIGURE 6)

The test solution contained 1 ml. of 2% starch, 5 ml. veronal buffer (pH 7.0), 1 ml. of the enzyme solution, and 3 ml. of water. This solution was brought to a temperature equilibrium of 38°C. before the enzyme solution was added. The amount of starch present was determined by the starch-iodine method at intervals of 15 minutes. By the decrease in starch remaining in the test solution, the amount of starch hydrolysis due to amylase was determined.

Time Interval	Mg. Starch Hydrolyzed		
	I	II	III
0	0.00	0.00	0.00
15	0.00	0.00	0.01
30	0.05	0.04	0.05
45	0.07	0.07	0.08
60	0.10	0.09	0.10

TABLE IX

PHOSPHORYLASE ACTIVITY ACCORDING TO THE METHOD OF GREEN AND STUMPF (19)

(FIGURE 7)

The test solutions consisted of 0.5 ml. veronal buffer of pH 7.0 (48), 0.2 ml. 5% starch, 1.0 ml. 0.1 M. glucose-1-phosphate, 1 ml. phosphorylase solution, and 0.8 ml. distilled water. The glucose-1-phosphate was added to the rest of the mixture after a temperature equilibration of 38°C. was attained. After 5 minutes the reaction was stopped with 5% trichloroacetic acid and the amount of orthophosphate liberated was determined according to the method of Sumner (59).

Test No.	Total mg. Orthophosphate Liberated Per 5 Minutes
1	0.3938
2	0.3940
3	0.3933
4	0.3938

TABLE X

THE EFFECT OF ORTHOPHOSPHATE, MONOFLUOROPHOSPHATE, AND FLUORIDE ON THE SYNTHESIS OF STARCH BY PHOSPHORYLASE

(FIGURES 10 AND 11)

The test solution contained 2 ml. of 1% glucose-1-phosphate, 4 ml. veronal buffer (pH 7.0), 0.1 ml. of 5% starch, 1 ml. phosphorylase solution, and varying concentrations of 0.1 M. solutions of either Na_2HPO_4, Na_2PO_3F or NaF. The final solution was at a constant volume of 10 ml. by the addition of water. The reaction was followed by determining the amount of starch formed by the starch-iodine method.

Molar Concentration of Na_2HPO_4	Mg. of Starch Synthesized*	% Inhibition
0.0200	0.00	100.00
0.0150	0.37	97.04
0.0100	1.89	85.25
0.0050	5.77	53.70
0.0025	9.10	27.00
0.0000	12.48	0.00

TABLE X

(continued)

Molar Concentration of Na_2PO_3F	Mg. of Starch Synthesized*	% Inhibition
0.0050	0.000	100.00
0.0040	0.615	95.07
0.0025	5.210	58.10
0.0010	9.530	23.50
0.0000	12.450	0.00

Molar Concentration of NaF	Mg. of Starch Synthesized*	% Inhibition
0,020	11.65	6.4
0.010	12.08	3.0
0.005	12.45	0.0
0.000	12.45	0.0

*These figures are an average of five runs.

TABLE XI

STANDARD PHENOLPHTHALEIN CALIBRATION CURVE

(FIGURE 12)

Reagents:

1. Stock phenolphthalein. 100 mg. phenolphthalein in 100 ml. 95% ethyl alcohol.

2. Glycine buffer. Dissolve 9.19 gm. glycine and 7.17 gm. sodium chloride in water. Add 15 ml. of concentrated sodium hydroxide (100 gm. NaOH in 100 ml. H_2O). Add water to 900 ml. Add 40 gm. sodium pyrophosphate. Dilute to 1 liter. pH = 11.2.

Tube No.	Mg. Phenolphthalein	% Transmission*
1	0.005	85.5
2	0.010	75.5
3	0.020	58.0
4	0.030	44.8
5	0.040	35.8
6	0.050	28.0
7	0.060	22.3
8	0.070	18.0

*These figures are the averages of five separate test runs.

TABLE XII

TIME CURVE FOR PHOSPHATASE USING SODIUM PHENOLPHTHALEIN PHOSPHATE AS SUBSTRATE

(FIGURE 13)

The reaction mixture contained 10 ml. of alkaline sodium phenolphthalein phosphate solution, 1 ml. phosphatase solution (1 mg./ml.), and 9 ml. of water. The amount of phenolphthalein was determined by the method of Huggins and Talalay (31).

Time Interval	Mg. Phenolphthalein Liberated Per Aliquot	Total Mg. Phenolphthalein Liberated*
0	0.00000	0.0000
5	0.00075	0.0075
10	0.00550	0.0550
15	0.01050	0.1050
30	0.02600	0.2600
45	0.03700	0.3700
60	0.04130	0.4130

*These figures are the averages of four separate test runs.

TABLE XIII

ENZYME CONCENTRATION CURVE FOR PHOSPHATASE

(FIGURE 14)

Each of a series of reaction mixtures consisted of 10 ml. sodium phenolphthalein phosphate solution 0.5, 0.75, 1.0, 1.25, 1.5, and 2.0 ml. phosphatase solution corresponding to 0.5, 0.75, 1.0, 1.5, and 2.0 mg. phosphatase. Water was added to make the volume 20 ml. The mixtures were allowed to react for 30 minutes.

Concentration of Phosphatase (mg./20 ml.)	% Transmission After 30 Min.	Total Mg. Phenolphthalein Liberated/30 Min.*
0.00	100.0	0.000
0.50	77.0	0.090
0.75	62.0	0.180
1.00	51.5	0.255
1.25	45.3	0.305
1.50	41.0	0.345
2.00	36.0	0.400

*These figures are the averages of four separate test runs.

TABLE XIV

THE EFFECT OF ORTHOPHOSPHATE ON PHOSPHATASE

(FIGURES 15 AND 16)

The reaction mixture contained 10 ml. sodium phenolphthalein phosphate substrate, 1 ml. phosphatase solution, varying amounts of 0.1 M. sodium phosphate and water to bring to a total volume of 20 ml.

Concentration of Orthophosphate	Total Mg. Phenolphthalein Liberated/30 Min.*	% Inhibition
0.00000	0.3925	0.000
0.00005	0.3830	2.420
0.00050	0.2520	35.800
0.00100	0.1750	55.420
0.00150	0.1054	73.130
0.00200	0.0944	76.000
0.00250	0.0904	77.000
0.00300	0.0644	83.600
0.00350	0.0548	86.050
0.00400	0.0440	88.800
0.00450	0.0396	89.900
0.00500	0.0356	90.900
0.01000	0.0134	96.600

*These figures are the averages of four test runs.

TABLE XV

THE EFFECT OF SODIUM MONOFLUOROPHOSPHATE ON PHOSPHATASE

(FIGURES 15 AND 16)

The procedure was the same as that for the determination of the effect of orthophosphate on phosphatase.

Concentration of Na_2PO_3F	Total Mg. Phenolphthalein Liberated*	% Inhibition
0.00000	0.4780	0.00
0.00025	0.4330	9.42
0.00050	0.3920	18.00
0.00100	0.3280	31.50
0.00150	0.2830	41.00
0.00200	0.2400	49.70
0.00250	0.1990	58.30
0.00300	0.1680	64.80
0.00350	0.1530	68.00
0.00400	0.1250	73.80
0.00450	0.1120	76.75
0.00500	0.1030	78.70
0.01000	0.0506	89.40
0.02000	0.0134	97.20
0.03000	0.0056	98.83
0.04000	0.0034	99.90

*These figures are the averages of four runs.

TABLE XVI

THE EFFECT OF VARIOUS CONCENTRATIONS OF FLUORIDE ON THE ACTIVITY OF PHOSPHATASE

(FIGURES 17 AND 18)

Molar Concentration Na F	Total Mg. Phenolphthalein Liberated*	% Inhibition
0.0000	0.425	0.00
0.0050	0.412	3.06
0.0125	0.410	3.53
0.0250	0.406	4.47
0.0375	0.395	7.06
0.0500	0.390	8.24
0.1000	0.378	11.06
0.1500	0.363	14.59
0.2000	0.330	22.36
0.2500	0.318	25.18
0.3000	0.275	35.30
0.3500	0.248	41.65
0.4000	0.225	47.06
0.4500	0.205	51.77

*These figures are the averages of four test runs.

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APPROVAL SHEET

The thesis submitted by Robert Aloysius Sliwinski has been read and approved by three members of the Department of Biochemistry.

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with reference to content, form, and mechanical accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements for the Degree of Master of Science.

January 21, 1953
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